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SIR STEWART DUKE-ELDER

Proctor Medal Award

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-ninth Meeting

Miami, Florida

June 13, 14, 15, 16, 1960

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The papers in this supplement have been selected and edited by the Editorial Board of the Association for Research in Ophthalmology and neither the American Journal of Ophthalmology nor the Ophthalmic Publishing Company can assume responsibility for their content or editorial style.

ON THE PRESENTATION OF THE PROCTOR MEDAL

Of the Association for Research in Ophthalmology to Sir Stewart Duke-Elder, G.C.V.O., F.R.S., etc., Miami, Florida, June 15, 1960

On the occasion of Stewart Duke-Elder's 60th birthday, The American Journal of Ophthalmology issued a *Festschrift* in his honor (**45**: April, pt. II, 1958). I think many of you have read it. You will then recall the biographical sketch of him written by me with a heart full of affection and good wishes. There is little need, therefore, to repeat here in all detail the fascinating biographical data of his career and his manifold contributions to our knowledge. But for those members of this Association who do not subscribe to The Journal and thus may not have read the sketch referred to, let me give you some slight idea of "What makes Stewart run."

He was born in Dundee, Scotland, in 1898, of godfearing and austere Scotch Presbyterian parents of limited means. His father was a minister in the Free Church. Stewart was raised in the spartonlike chill and theologic gloom typical of the times. Fortunately this did nothing to curb his blithe spirit which was undoubtably fortified by the dour challenges. For even in those early days his alert mind and high, even rebellious, spirits became manifest. He captured every prize and gold medal in his school days and also did his share or more, of mischievous extracurricular activities. He went on to the United College of St. Andrews, graduating in Arts and Medicine in 1922. He earned the degrees of B.Sc., M.A., M.B., Ch.B., M.D. (gold medal) and D.Sc. all at St. Andrews. In 1925 he earned his Ph.D. degree in the University of London. He received many collegiate honors of various sorts, and preferred the companionship of his many friends and classmates in convivial activities, to long and arduous hours spent on the adjacent golf courses.

With diplomas and medals in his otherwise nearly empty pockets, he went up, as they say, to London, something like David Copperfield, to seek his fortune. He had to stop on the way at a town near Northampton where he served a short time as a Locum Tenens to earn enough guineas to continue his journey to St. George's Hospital in London. Wise, energetic, ambitious but above all blessed with genius and the spirit of hard work, in a year or so he received an appointment as junior ophthalmic surgeon and took his F.R.C.S. in 1924. He soon attracted the attention of that very great ophthalmologist and scientist, Sir John Parsons whose favorite pupil he quickly became. On Sir John's death in 1957, Stewart in a moving tribute said, "To him I owe everything; he taught me ophthalmology and made possible anything I may have done professionally."

Some of you remember the obscurely erudite book, *An Introduction to the Theory of Perception*, by Sir John Parsons, published in 1927. In presenting the Howe Medal of the American Ophthalmological Society to Sir John in 1936, Frederick Verhoeff said that this book of Sir John's was like Browning's poetry, only the author and God could understand parts of it, and sometimes God was at a loss. But he forgot or did not know then of Stewart, for Stewart not only understood it, but in later life he capped it with the extraordinary Volume I of his new System of Ophthalmology 1958, *The Eye in Evolution*. He has fulfilled the prophecy contained in the last sentence of Sir John's book which says ". . . the knowledge which we gain from experimental and comparative physiology, controlled and judiciously interpreted in the light of psychological analysis of our own conscious processes, has revealed much and is destined to reveal more."

I dare say if pressed right here and now, Stewart could give us a clear exposition of Einstein's theory so that even we could understand it.

There is no further need to tell you the onward and upward progress of this magnus Apollo from 1924 to the present, for time and space, even in the sense of Einstein, would not permit us to give all of the facts. Besides most of you know of and employ the

many contributions Stewart has given us, from basic research in the laboratory to clinical observations and research in the wards; from the care of the rabbit to the care of the Babbitt. His textbook is the Bible of all students and practitioners of ophthalmology who can read English. This man has given us all by himself more scholarly, instructive and lucid words in all branches of ophthalmology than has the French Academy for the French language or Toynbee and Gibbons for the study of history.

Oliver Goldsmith once said:

"And still they gazed and still the wonder grew
That one small head could carry all he knew."

He has received many, many richly deserved honors of great significance in the world of medicine and science. The latest and I think one of the most pleasing to him, is his recent election to Fellowship in the Royal Society. This is the oldest scientific society in Great Britain, and one of the oldest in Europe and the world. Its full title is "The Royal Society of London for Improving Natural Knowledge." It was sanctioned by Charles II in 1660, six years before the *Annus Mirabilis* of war, plague, and fire, poetically described by Dryden.

At the present time, Sir Stewart is one of but four physicians who are Fellows. The significance of this, it seems to me, is that it illuminates all ophthalmology. I don't know how many ophthalmologists have become members of this august organization. I know that Sir William Bowman and Sir John Parsons were, but I am sure that the number is few, and in these days when the pendulum of prestige has swung from the clinic to the laboratory, there would be little chance for a physician to become a member, unless his contributions were overwhelmingly of scientific importance in research, as in the present instance.

As you see Sir Stewart is slightly bent over. He is not, as Chaucer has said "somdel stoppen in age." On the contrary this posture is, I am sure, due to the weight of the numerous medals on his chest and the felicitous burden of the pack of honorary academic hoods of every color and the postnomine initials and titles that he totes on his back.

We are happily about to add to this burden and award him the Proctor Medal of this organization, as a token of appreciation of him and for his works. But before doing so I should like to describe one other honor given him prior to the First Congress of the European Society of Ophthalmology recently held in Athens. So far as I know it is a unique honor and one that will probably never be given again.

The scene was Olympia in Greece, the site of the original olympic games, the sacred torch and the hallowed grove containing the temple of Zeus, at whose altar the champions of the world of sport, philosophy, science, art and medicine were annually crowned with the divine wreath, made of the leaves of the holy kailistephanos laurel.

Assuming the authority by right of locus pocus of the world of ophthalmology, John H. Dunnington and I crowned Stewart at the site of the blessed altar, "World Champion Ophthalmologist," by placing the laurel wreath upon his brow and in the presence of many witnesses for the most part tourists to be sure and to the unheard thundering applause of all of the occult spirits of the gods of Olympia.

At this moment, as you can see by the picture, the sun came from behind the clouds and most lustriously gave its benediction. We can attest too, that innumerable song birds burst into celestial music and the branches of the ancient cedars bowed in the wind in homage to our champ.

Stewart, adding to the uncountable honors, diplomas, degrees, medals, certificates, placques, hoods and testimonials you have already most justly and properly received, the Association for Research in Ophthalmology has given me, a devoted old friend of yours in war and peace, the honor and deep pleasure of bestowing its highest award, the Proctor Medal, upon you as an outward sign of overwhelming inward appreciation for all that you have given us of yourself. We are positive that none of your honors has been given more generously and wholeheartedly, or with deeper affection, in more sincerity or with more acclaim.

Derrick Vail, M.D.

Champion ophthalmologist of the world congratulated by his lady.

COMMENTS ON ACCEPTANCE OF THE PROCTOR MEDAL

SIR STEWART DUKE-ELDER

I think perhaps the most clamant impression that this Award brings to me is the fact that the America I once knew has experienced in so short a time a revolution of such magnitude as to make the existence of this Association and the Proctor Medal itself possible. You must remember that my first ophthalmologic pilgrimage to America occurred more than 30 years ago. At that time you certainly had great clinicians and surgeons in our specialty, men whom I knew well, whom I respected and liked such as Wilmer and deSchweinitz and John Wheeler. These in your New World were peers of any in the Old World. But at that time there was little of organized research. There was Frederick Verhoeff working by himself in a small laboratory at Boston; there were Alan Woods and Jonas Friedenwald at Baltimore; but no others did I find. Alan Woods, by the award of the Gonin Medal, justly received the highest honor international ophthalmology can give; Jonas Friedenwald, although not so well known personally abroad, was equally respected on either side of the ocean and equally loved by all who had met him. But I think too few of us realize that the pioneer of ophthalmologic research in America as we know it, the first in this country to live primarily for his laboratory, was Frederick Verhoeff.

The extraordinary thing is that I am speaking of only 30 years ago. How changed is it today! With the vast weight of its population, the fertilization resulting from its boundless hospitality in receiving the homeless and often the best from other lands and its almost unlimited material resources, the United States has been able to stage a resurgence of research—and that not only in ophthalmology—which in its scope, its extent, and its excellence has been equalled by no country in the world. An association like this, vital, flourishing and enthusiastic, is unique. For this to occur within a single professional lifetime is a phenomenon in the history of ophthalmologic progress. But unique phenomena—whether they concern ophthalmology or sending satellites to Venus —are a commonplace in this country.

In this development I can see an interesting and perhaps natural evolution. It may be a result of the history of the expansion of your country, of the expenditure of your national energies in the very practical and urgent task of the pushing out of frontiers and the opening up of vast tracts of virgin territory rather than in the consolidation of limited resources, that hitherto the greatest strength of American research in ophthalmology—and I think possibly in other things —has been in the successful application of knowledge rather than the creation of its fundamental basis; its metier lies in "know how" rather than "know why." In my country where academic research is old, the highest prestige has gone to the pure scientist; you have been more practical in your philosophy. Perhaps it is fortunate that this is so, for while basic research is usually individual and demands little in the way of expenditure, its successful application requires elaborate team work and the expenditure of enormous resources. As a corollary, however, while some prestige may accrue from the former, practical rewards and popular acclaim in abundance flow from the latter. Today basic research is beginning to occupy your attention to a progressively greater degree. In another 30 years' time, although I shall not be here to see it, the activities of this Association promise to be so rounded and complete, philosophically and technologically, that to award the Proctor Medal will be indeed a problem demanding the highest discrimination.

GAUDEAMUS IGITUR DUM IUVENES SUMUS

SIR STEWART DUKE-ELDER, F.R.S.
London, England

Many of you may wonder at my choice of the title for this lecture. You may think that in this apparent reference to my student days I am a candidate for Korsakoff's psychosis. But I am speaking not personally but of us all as an ophthalmologic community when I say "Let us rejoice while we are still young." Some 400 years ago Ambroise Paré, one of the earliest and one of the greatest of the many illustrious surgeons which France has produced, declared on the publication of his great text-book of surgery—the first really great classic in that science in what, if we take a historical perspective, we might call modern times—that nothing was left for posterity to add to what he had written. It is because Ambroise Paré was wrong that we have reason to rejoice.

In no branch of medicine is the reason for rejoicing stronger than in ophthalmology. So far as research is concerned we are now adolescent—but only just so. It is true that diseases of the eye were studied and treated five or six thousand years ago in the early Assyrian civilization of Mesopotamia; it is true that oculists flourished in the civilization of Ancient Egypt and in the era of classic and Arabic learning; but as a scientific specialty it may be said to have been born a little over a century ago at the time of Helmholtz and von Graefe, of Donders and Bowman, at the time when a great rationalizing process spread throughout the whole of medicine, when the spirit of observation and experiment replaced the doctrinal theorizing of Galen and Paracelsus. Since then, for 100 years ophthalmology has been developing and exploiting the methods used by these giants of one hundred years ago, observing ocular diseases clinically and pathologically, interpreting the changes occurring therein in terms of the cellular pathology of Virchow, the bacteriology of Pasteur and the physiology of Claude Bernard.

For 100 years the impetus given by this new knowledge has evolved and progressed. A multitude of diseases of the eye has been described and their cellular pathology elucidated. In a large number of conditions changes in the eye have been associated with disturbances elsewhere in the body and their relationships established. But in most cases the techniques and the methods which have been evolved during the last century of medical progress do not take us very far. We recognize diabetic retinopathy, for example, we know the evolution of its clinical picture, we are fully aware of its gross pathology; but we do not know why it appears and we stand helplessly by, impotent and unable to do much while our patient goes blind.

Similarly, surgical craftsmanship has gone on apace on the same general lines as were laid down by von Graefe 100 years ago although with vastly greater technical achievements. Today we can renew a scarred and opaque cornea by the graft of a clear one; we can remove a cataract and if we are very bold, replace it by a plastic lens in the anterior chamber; we can extract a diseased vitreous and replace it with a substitute. Indeed, it would appear that if a little piece of retina survive we can technically reconstitute most of the eye. But this is only craftsmanship; and, working without more fundamental knowledge, we are often defeating our own ends as we run against forces we do not understand.

We can graft a new cornea, but it sometimes goes opaque, why we do not always know. Indeed, we do not really know why a normal cornea remains transparent nor the fundamental reasons why it goes opaque. We suspect that a cataract is essentially due to a disturbance of the enzymatic activity of the lens; we can remove it with ease and often with elegance, but we cannot control the factors which cause its formation. Al-

though we can do some effective plumbing to restore an obstructed circulation of the intraocular fluid in glaucoma, we are still disputing as we search for the primary cause; we can do little to prevent its onset and sometimes, despite our plumbing, our patients go blind.

If we are to make further progress a new approach is needed; and let us rejoice that it is well under way. I do not think for one moment that progress on the old established lines has ceased; I think that clinical observations will bring to light many new relationships, that the microscope will reveal many new facts in pathology; and I do not think surgical techniques have reached their practical limits. Nor do I think that research in the laboratory can realize itself unless it is correlated with work in the clinic. But if we are to make great new advances, if, instead of progressing by evolution we are to effect the same type of revolution as occurred a century ago when Darwin revolutionized the whole of biologic thought, we must enter with enthusiasm into the almost boundless opportunities that are opening up today when an analysis of structure is no longer confined to the study of cells and the gross constituents of cells, but extends with the aid of the electron miscroscope to the molecules which make up these constituents; when an analysis of function extends, for example, to the introduction of agents by electrophoretic injection with microcapillaries into a single cell or a colony of cells. No longer are we content to describe a disease in its gross effects and look upon it as known if we can place it in the most suitable pigeon hole in a system of classification; we have begun to look beyond the picture of cellular pathology which represents merely the ruins disease has left behind, and to seek instead the cause of the initial defect; to interpret changes not in terms of structural damage but rather in terms of biophysics and biochemistry—not at the level of cellular events but in terms of intracellular events, at the level of the forces which determine the reactions within the cells.

Let me illustrate this by two examples, one from physiologic and one from clinical ophthalmology.

For over a century much thought and work have been expended upon the structure and function of the retina and the visual pathways. Since the time of von Kölliker and Heinrich Müller we have known of the microscopic appearance of the retinal layers, and a host of neuroanatomists have elucidated the gross outline of the visual pathways. Knowledge of the electrical phenomena of the retina dates back to the time of Du Bois Raymond more than a century ago, but we still do not know the significance of the changes of potential that constitute the electroretinogram. We know nothing of how the photochemical events caused by light in the visual cells are converted into neural energy, we know little of the retinal metabolism, and we even dispute whether the analysis of color vision is a peripheral or a central phenomenon. Incidentally, I cannot help thinking how fortunate we are that a man with the erudition, the industry and the ability of Polyak was vouchsafed to our generation; for in his classic book published posthumously, he gave a magnificent synthesis of our knowledge of the anatomy and physiology of the visual system in the first stage of its development.

The next such book will be vastly different. We are now beginning to learn of the ultrastructure of the visual cells with their unique organization; we are able to locate the key enzymes within these cells and in the retinal layers, and the glial tissue which until lately we had conceived merely as a passive scaffolding we now suspect to be of great importance in supplying the retina with its requirements of energy. With metabolic poisons such as iodate and iodoacetate we can eliminate particular elements or alter their activity by the microinjection of drugs into a particular layer and thus relate structural organization and activity. New techniques are rapidly opening up new horizons with almost bewildering rapidity, which promise to allow us to hope that a wide

"break through" may be possible in our knowledge of the neural basis of why and how we see.

What is the meaning of Maturana's studies in the optic nerve with the electron microscope? The immense numbers of fine non-medullated fibres that he found in the optic nerve of the frog—and his pictures are convincing—would seem to indicate that our previous microscopic estimate may have to be multiplied by a factor of 30. If perchance there are not one million but 30 million fibres in the human optic nerve, what do they do and where do they go? It would seem that visual physiology may need to be revolutionized. For decades it has been accepted that, apart from the pupillary pathways, the lateral geniculate body was the essential primary station for the optic nerve fibres and that it acted merely as a passive transmitter of visual impulses. What does the spontaneous electrical activity of the lateral geniculate body mean, itself dependent on the hypothalamus and the reticular formation which appears to exercise so marked an influence on retinal activity as a centrifugal feed-back mechanism? What of the collateral optic pathway to the superior colliculus? What is the meaning of the topographical point-to-point representation of the retina on that body shown by Julia Apter with its presumptive phototactic implications? What of the control of the intraocular pressure by the hypothalamus and the relationship between the basic physiology of the eye with this area and the whole limbic system of the brain? It may be that the complex autonomic nerve supply to the retina, the pigmented epithelium and the entire uveal tract may be of more importance than we have thought. It is true that today we recognize the importance of the eyes in the governance of the posture of the body; but it may well be that in the future we will marvel at the limitation of our present horizon when we conceive of the eye as a visual mechanism and pay little attention to its fundamental relationship with autonomic activities.

As a clinical illustration of the new vista in research that is opening up, I will outline the story of the treatment of organismal infections during the last 100 years. During the second half of the last century a vast number of bacteria has been discovered and labelled as the cause of disease; during the first half of this century an almost equal number of viruses. The first microorganism to be discovered was by Pasteur almost a century ago in 1863 and it is fitting and delightful that it was the organism that causes the souring of wine; the last to be isolated were the viruses of trachoma and inclusion conjunctivitis.

A century ago Lister, looking for a method to kill those minute organisms which he had concluded were the cause of suppuration, learned by chance (and that is important) that the municipal authorities of the English town of Carlisle successfully treated their sewage waste by mixing it with creosote. From this he evolved the technique of antisepsis by carbolic acid; if he coagulated the tissues of the host and killed the cells at the same time as he killed the invading microorganisms, that was unfortunate; but not nearly so unfortunate as the lingering death by suppuration and amyloid disease that was the almost invariable sequel of any major surgical intervention at that time, and converted the hospitals of a century ago into charnel houses to enter which usually meant death. Towards the end of the last century a new stage was introduced by Paul Ehrlich who, working on the new science of histologic staining and observing the high affinity of certain dyes for certain constituents of the cells, tried deliberately to find a drug that would kill the micro-organism and leave the host intact, a *therapia magna sterilisans* after the manner of the alchemists. Thus, with all the resources of the dye industry of Germany at his disposal, at the 606th attempt he achieved the arsenical cure for syphilis. Another chapter was begun by Alexander Fleming who noted, again by chance, in 1928, that a penicillin mold in-

hibited bacterial growth on his culture plates. This observation opened up the possibility of exploiting for our own ends the ceaseless struggle in which organisms can survive only by preying upon one another. As a result, over the last decade whole armies of research workers, backed by all the resources of the now immense pharmaceutical industry, have extracted countless molds from the soil or the bodies of bacteria, searching endlessly for antibiotic drugs which will immobilize more and more organisms and render them ineffective.

But the important thing to remember is that up to the present time little has been known of the mode of action of these antibiotics. They are searched for empirically, by a process of trial and error wherein many are called but few are chosen. In one investigation 10,000 actinomyces from the soil were studied, 2,500 were found to produce antibiotics, 10 of them were new, and of the 10 only one was clinically effective. This is a passive process in which we do not really determine the course of events. There is no real revolution of method here; the process is essentially the same as that used by Lister.

I am not suggesting that this chancy method of pharmacologic development has not paid dividends; nor have the dividends been confined to recent years and antibiotic drugs. There is, for example, the apocryphal story of the introduction of quinine into medicine—how in an earthquake in Peru a number of trees were uprooted into a lake of the water of which an Indian, urged by his thirst in an attack of ague, drank and was cured, so that the Countess of Chinchona, herself similarly cured, brought the infusion of Jesuit's bark to Europe. Or the true story of the English parson, Edmond Stone, who, tasting the bark of the English willow tree in Oxfordshire, found that it cured his ague in 1757, so that the salicylates were introduced. Or again, that of William Withering who found that the old women of Shropshire in England used a decoction of foxglove leaves as a cure for dropsy and thus introduced digitalis into our therapeutic armamentarium in 1875.

But today a new science of microbiology is growing up in the study of the intermediate metabolism of the microorganisms themselves, and we are beginning to see the weak spots in their armour through which they can be attacked—not passively but deliberately. We now think of the action of penicillin in terms of the specific disorganization of the growth of the cellular walls of bacteria, perhaps by blocking the metabolism of muranic acid peptide, or of the broad-spectrum antibiotics in terms of the inhibition of the synthesis of specific bacterial proteins. We can now synthesize the nucleus of the penicillin molecule and deliberately add to it side-chain after side-chain so that this or that resistant bacterium can be successfully attacked. This is a new phase, the potentialities of which are difficult to assess, a phase wherein we study the chemistry of the organism's metabolism and deliberately introduce some synthetic agent that will bring it to a stop.

By these means we have already overcome most straightforward bacterial infections, but we are still left with the smaller viruses which lead an intracellular parasitic life. With our present knowledge, lying within the cell they are safe—at any rate the smaller and more elementary ones—and cannot be destroyed except by methods which will destroy the cell itself. It is unfortunate that in ophthalmology virus infection is so common; 40 percent of external infections of the eye in Great Britain are due to viruses and in this class of disease the virus of herpes simplex causes more visual disability than any other single agent. The attack on these will probably have to wait until our knowledge of intracellular chemistry and the metabolism of viruses is further advanced than it is today. But all things are possible.

It would seem that a virus, like the chromosomes in a cell, is composed of a central double helix of nucleic acid arranged

in spiral fashion, attached to the outer aspect of which are many thousands of protein molecules. It has been conclusively shown that the ribonucleic acid contains within itself the genetic code for these organisms and would seem to be the sole infective agent; it controls the synthesis of the protein which in turn controls the activity of the enzyme systems of the cell so that these essential elements in the cell's metabolism are diverted from the purposes of the host to those of the parasite. Burnett has put it well when he suggests that the best way of regarding the virus-cell relationship is as the intrusion of one genetic system into another.

But there is new light on the horizon. Ribonucleic acid with its endless repetitions of four main purine or pyrimidine bases—adenine, guanine, cytosine, and uracil—has now been synthesized. From the practical point of view it would seem that if the orderly replication of the nucleic acid chain could be disturbed without intefering with the somewhat different chains of deoxyribonucleic acid in the actively reproducing cells of the body, the virus would be precluded from proliferating without damage to the host. Already a series of purine-acid pyrimidine antagonists have been prepared which could act either by modifying the individual groups in the nucleotide units or by blocking the synthesis of a necessary precursor of a rapidly proliferating virus. Plants can be protected against the cucumber virus in this way, and very recently it has been shown that hydroxylamine can inactivate the ribonucleic acid of certain encephalomyelitic viruses, the fowl-plague virus and the swine-influenza virus. Indeed, research is progressing so rapidly on these and kindred questions that it may well be that the time is not far distant when virus diseases will become amenable to the same type of control as bacterial disease. It is conceivable that the same method of attack could be made to control the uncontrolled proliferation of cells in cancer. But that time is not yet come; it still lies in the future—but we are yet young.

This then, as I see it, is a picture of the present; an evolution which has lasted for a century is breaking out into a revolution today. What is to happen tomorrow? If I were to attempt to forecast whither ophthalmology is going in this exciting stage of its affairs, I would divide it into two sections. In the first it is probable that ophthalmology, because of the poverty of its resources, will wait upon advances in general medicine. This includes such subjects as immunity and autoimmunity, the treatment of organismal infections and allergies, and the multitude of the ocular manifestations of general disease. The task of our specialty will be the recognition of what is germane and its application to the problems of the eye; but because of the increasing complexity of biologic science and the consequent departmentalization of medicine, this will not be easy and must be done purposively.

The second type of problem is peculiar to the eye itself; and here we must help ourselves. This concerns the peculiar pathologic conditions which are encountered in the cornea, the lens, the vitreous and the retina, each of which presents individualistic reactions whether of an edematous, inflammatory or degenerative nature. Today, despite the passage of 100 years, our knowledge of the metabolism of these tissues is extraordinarily crude. We may suspect that the transparency of the cornea is based on the unique regularity of the arrangement of its fibrillar structure, but regarding the metabolic activity which maintains its state of hydration and transparency, we are essentially ignorant. We know the gross end results of the denaturation of the proteins of the lens that appears as a cataract, but what initially goes astray in its enzymatic activity that eventually ends in the death of its cells is yet a matter of surmise. The mechanism of the maintenance of the vitreous gel may well be an inter-relationship between its hyaluronic acid component and its protein fibrillar scaffolding, but what circumstances maintain and what disrupt this relationship are not yet clear. When it does become clear

we will know why a vitreous becomes fluid or endogenous opacities form and we will understand more clearly why a retina becomes separated. The curious manifestations of the retinopathies which accompany general diseases as diabetes, hypertension and nephritis, the initial formation of microaneurysms and the subsequent evolution of the changes in this tissue that lead so often to blindness are so peculiar and individualistic, so different from the manifestations of these diseases in other organs and so apt to occur in conditions confined to the retina alone—all these facts lead to the conclusion that their incidence and characteristics depend on the unique metabolism of this tissue and its intense and precariously poised glycolytic activity. When we know more of this fascinating problem we might conceivably make a real therapeutic advance; but until we do, this can merely be a matter of chance which we have no right to expect to come spontaneously to our assistance.

Our great problem today is, therefore, a lack of understanding of the basic physiology of these ocular tissues; and it is interesting that they all, with the single exception of the inner layers of the retina, are avascular tissues—a problem about which comparatively little is known to general physiology and biochemistry.

It is here, I think, that the great opportunity lies for ophthalmology in the immediate future—to attack the first principles of normal physiology rather than the end results and tissue ruins of pathology. Fortunately the techniques becoming available now in the new revolution that is occurring throughout biology make it seem reasonably possible that the opportunity can be exploited. The craft of ophthalmic surgery is dramatic, glamorous and immediately rewarding—and it is also easy. But we are at the stage today when, instead of devising new techniques to make the success of a corneal graft more certain, a cataract extraction more elegant or the sutures of a wound more complicated, we should be thinking in terms of preventing the cornea or the lens from going opaque. It is a very little thing to be able to say that the immediate cause of simple open angle glaucoma is an obstruction to the outflow channels of the aqueous —even a measurable obstruction which can be expressed in pseudo-mathematical terms —or that the cause of closed angle glaucoma is a blockage at the angle of the anterior chamber; it would be better if we were in a position to say what caused the obstructive changes in the first case and the turbulence in the diencephalic control of the homeostatic mechanism that maintains the intraocular pressure at an even level in the second. We must replace the present day worship of technical "know how" by the more fundamental question of "know why."

I would not give the impression that I imagine for a moment that we are on the threshold of the revelation of all the secrets of ocular physiology and therefore, as a consequence, of ocular pathology. All I am saying is that the events and the potentialities of today make some of us regret that we are old and should make others rejoice in being still young. But, at the same time, we must remember that there are almost certainly many things that seem beyond the knowledge we can anticipate today, even in our most enthusiastic moments.

There are, for example, the genetic constitution with which we are endowed and the hereditary traits which may be present at birth or appear in later life. It is generally agreed that gross anomalies cannot be cured or modified except insofar as the carpentry of surgery can alleviate the deformities they cause. But if we look into the future it may not be conceivably impossible that we may eventually be able to do something about hereditary defects—if and when we know more of their nature. As in the case of viruses, it is now established that the essential factor determining the genetic code through which our hereditary constitution is handed down is the immensely long double spirals of deoxyribonucleic acid in the chromosomes; this, as we have said, determines the nature of the associated protein

which acts as the structural mold determining the pattern of intracellular enzymes and thus controlling the ordered sequence of chemical events which constitutes the cell's activity.

This may sound simple: but it is to be remembered that a coded repeat of the four different components of the nucleic acid complex in a relatively small chain of 100 units could result in 10^{97} different arrangements —a number very much larger than the total of known living organisms on earth, in the air and in the oceans. We need not therefore fear that we are made to measure like a massproduction automobile. There is ample room for the code of every living thing to be different and individualistic. And the system of chromosomes which form the genetic apparatus in each cell in man is probably made up not of 100 but of about three thousand million links arranged in an unknown number of DNA chains, so that we need not fear any difficulty in realizing our individuality.

It is now generally agreed that congenital or hereditary defects (as well, incidentally, as some of the changes which characterize evolutionary progress) are determined by the chemical changes known as mutations in the molecules of DNA in the chromosomes. We can already speculate on the chemical basis of these changes that lead to errors in the replication of nucleic acid, whether they be random changes at one point in the molecule or are due to a failure in the availability of an enzyme system necessary for its synthesis, brought about by environmental influences such as damage by radiation, competition with a virus or combination with a carcinogen. Whatever be the mechanism, if the gene (or is it now a cistron?) suffers mutation, the enzyme for which it is responsible is changed in its reactions, or is lacking. We are far from understanding the intimate mechanism of any of these things today; but we know some gross facts, for example, that albinism is due to the lack of one of the enzymes required to convert the phenylalanine derived from the food into melanin; or that Tay-Sachs's disease is due to lack of the enzyme necessary to oxidize sphingomyelin; or alkaptonuria to lack of the enzyme necessary to oxidize homogentisic acid. Even when it is determined by heredity, diabetes can to some extent be controlled by insulin, and the pioneering attempts of Falls in developing a certain amount of pigmentation in albinos after the exhibition of tyrosine may be taken as a portent that in the future this approach, even although very far off, may not be altogether impossible. And it seems less impossible today when only yesterday DNA molecules have been synthesized in the test tube. If, as Zamenhof has remarked, these synthetic molecules carry the genetic code of their natural counterparts, the reproduction of genes in the test tube is a fantastic if not a frightening achievement.

Finally we come to the vast and incapacitating group of degenerative conditions. Since the gross evil effects of bacterial infections have been so largely controlled, the greatest amount of blindness in advanced countries is due to those degenerative conditions which, from adolescence until death, slowly and inexorably increase with the wear and tear of life. Many of the senile chorioretinal degenerations; the many pathologic results of vascular sclerosis, cataract and probably simple glaucoma, come into this category. The understanding of these is far off, their rational prophylaxis beyond our capacity. For they are biologically determined and are part of our genetic constitution. As Crew once pointed out, alone to the unicellular organisms which perpetuate themselves by binary fission has the gift of physical immortality been given. All multicellular organisms, including man, which experience the fulfillment of sexual reproduction, must pay a price for their satisfaction and evolutionary progress so that each individual is replaced by one more fully adapted to a changing environment; and that price is degeneration, decay and death. The aim of medicine is the banishment of untimely decay and death arising from environmental

causes so that each individual may make his full contribution to our community. His last —and not his least—contribution is acquiescence in timely death; and all medicine can do to help him is to make the processes of decay less ugly. And, indeed, it may be a good thing that we who, in the physical world in other things than medicine, use the stupendous material power we have with such improvidence and irresponsibility, should not be the sole arbiters of all the processes of birth and life and death.

These then are prospects of our future as I see them; but I would repeat, if we are to realize them, the laboratory must be married to the clinic. There is a tendency today to deify the laboratory worker and to accept his abstruse terminology and elaborate formulas as indisputable and almost divine. But these are meaningless unless they are interpreted in biologic terms; and those who will be the leaders in the new ophthalmology of the next century of progress will—like their predecessors of old—be men and women capable of analyzing, criticizing and weighing the significance of the findings of the scientist, appreciating their biologic limitations and applying this knowledge to the sick patient in the ordinary practice of everyday life.

THE MEASUREMENT OF RABBIT CILIARY EPITHELIAL POTENTIALS IN VITRO*

JAMES E. MILLER, M.D., AND MARGUERITE A. CONSTANT, PH.D.
St. Louis, Missouri

Indirect evidence indicates that a potential gradient may exist within the ciliary epithelium. Anionic and cationic dyes were observed by Friedenwald[1] to be transported in a selected direction. This process was dependent on the maintenance of a redox potential across the epithelium. Becker and Constant[2] were unable to demonstrate an effect of acetazolamide using the dye method of Friedenwald.

In addition, Holmberg[3, 3a] has observed by electron microscopy that alterations appear within the cellular membrane after acetazolamide administration. A marked increase in ultramicroscopic vacuoles was also noted in the nonpigmented epithelium of the rabbit.

It was thought that microelectrodes would offer a method of determining the existence of such a gradient. The factors producing alteration of the potential could be determined as well. This report concerns the intracellular potential measurements from the ciliary processes of normal rabbits in vitro and the changes of these potentials by in vivo and in vitro procedures.

While these studies were in progress, the findings of Berggren[4, 4a] with a similar technique became available. His results are in good agreement with the reported study concerning potential values, distance traversed, stepwise progression, cyanide and pH effect.

METHOD

Male rabbits weighing 2.0 to 2.5 kg. were used. These animals were fed Purina Rabbit Chow without antibiotics or animal factor, ad libitum. For starvation experiments all food was withdrawn for 48 hours or longer. The animals were anesthetized with sodium phenobarbital or killed by intracardiac air injection, depending on the requirements of the experiment.

The eye was enucleated and cut into two

* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine. This investigation was supported in part by a research grant, B-1349, from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

portions about the equator. The vitreous was carefully removed from the anterior half and radial incisions were made just peripheral to the ciliary body. The anterior portion was mounted in a plastic holder with the ciliary and lens surface facing upward. The presence of the lens and zonular fibers stabilized the ciliary processes for punctures. This preparation was then placed in 50 cc. of Tyrode's Solution.* The container, in turn, was surrounded by a water bath which was maintained at 34 to 38°C. A 15-watt bulb was used to maintain temperature and also acted as a source of illumination. Oxygen was forced through the media by the use of a polyethylene catheter.

The electrical potential was obtained by piercing the ciliary epithelium with a KCl filled glass microelectrode and the average of at least 25 acceptable potentials were obtained in all steps of the experiments. The electrodes were pulled from 1.5 mm. diameter Corning No. 9530 melting point capillaries, in a pipette puller constructed after the design of Becker.[5] Numerous tapers were used and the best electrode appeared to be one that tapered very rapidly as recommended by Frank.

The electrodes were stored in air in a closed jar until the day before use at which time the tips were inserted into a 3.0 M potassium chloride solution and the tops partially filled with distilled water. At the time of use the remaining distilled water was removed and the electrode completely filled with a 3.0 M potassium chloride solution using a 32 guage two-inch hypodermic needle. Any remaining air bubble was manually removed with a 75 micron stainless steel wire.

The electrodes were not used after 24 hours since storage of several days in potassium chloride solution increased the tip size of the electrode. This change in size produced indistinct cell borders and potentials of lower values which declined rapidly.

Gross manipulation of the electrode in three directions was accomplished by a three-way rack and pinion system. Fine movement of the electrode in a vertical direction was acquired by a hydraulic system using two syringes connected with rigid plastic tubing and filled with mineral oil. The syringe that moved the electrode was of a 2.0 cc. type with a diameter of 9.0 mm. An alligator clip was attached to the plunger and its jaws were covered with rubber tubing to hold the microelectrode. The second syringe was of a tuberculin type with a diameter of 3.8 mm. The plunger was spring loaded and controlled by a micrometer so that advancement of one micron could be obtained. The potential was recorded by two Ag-AgCl electrodes, one inserted into the media, the other into the glass electrode. The leads from the two silver electrodes were connected to a cathode follower, which in turn fed into a low-level DC Grass Polygraph with an ink writing system.

The input impedance of the cathode follower was 90 megohms. The resistance of the microelectrode could be determined by shorting a 50 megohm resistance across the input and observing its influence upon a 30 mv. calibration voltage.[6] The electrodes used measured 10 to 15 megohms in impedance and the tips were less than one micron in size since they could not be visualized at 450× magnification. The calibration and resistant measurements were also used as a method to determine breakage or obstruction of the electrode during the experiment.

The experiments were conducted so that the first eye was used as a control. The second eye was utilized to determine the influence of various agents administered in vivo. Alteration in potentials from agents used in vitro could be demonstrated in either eye.

* Regular Tyrode's Solution—1 liter: NaCl 8.0 gm., KCl 0.2 gm., $CaCl_2$ 0.2 gm. $MgCl_2 \cdot 6H_2$ 00.1 gm., NaH_2PO_4 0.05 gm., $NaHCO_3$ 1.0 gm. and glucose 1.0 gm.

Bring up volume: pH was adjusted to 7.4 by bubbling through CO_2 (30 to 45 minutes). (1) Glucose free. (2) Carbonate free—omit $NaHCO_3$. Do not bubble CO_2 through solution. pH 6.5.

TABLE 1

Experiment	Nonpigmented Epithelium	Pigmented Epithelium	Stroma
	(S.D.)	(S.D.)	(S.D.)
Normal (30)	27.8 mv. (3.1)	55.6 mv. (4.4)	5.1 mv. (1.8)
Controls (5)	28.4 mv. (2.3)	58.3 mv. (2.5)	5.6 mv. (3.1)
Acetazolamide in media	27.0 mv. (3.0)	55.0 mv. (2.8)	4.0 mv. (2.9)
Acetazolamide in vivo	22.9 mv. (4.2)	56.6 mv. (5.9)	6.3 mv. (7.5)
Controls (7)	27.8 mv. (4.9)	58.7 mv. (5.0)	5.5 mv. (2.1)
Glucose I.V.	23.5 mv. (4.2)	47.6 mv. (5.5)	6.6 mv. (3.7)
Controls (5)	27.5 mv. (1.8)	52.2 mv. (2.6)	4.0 mv. (1.1)
Sucrose I.V.	27.9 mv. (1.2)	55.0 mv. (0.2)	4.7 mv. (1.1)
Controls (5)	28.3 mv. (5.9)	61.7 mv. (4.8)	5.5 mv. (2.3)
Glucose I.V.	25.1 mv. (8.9)	47.6 mv. (5.5)	6.6 mv. (3.7)
Insulin in vitro	26.7 mv. (4.1)	59.4 mv. (2.2)	5.7 mv. (3.5)
Controls (4)	26.7 mv. (0.8)	52.2 mv. (1.2)	7.0 mv. (0.1)
Glucose and insulin I.V.	25.8 mv. (2.8)	55.9 mv. (0.6)	7.3 mv. (1.3)
Starvation (5)	29.6 mv. (2.1)	59.7 mv. (3.4)	6.0 mv. (2.1)

Results

A histologic cross section of the rabbit ciliary process indicates that variations in epithelium are found (fig. 1). It can be seen in some areas that a flattened epithelium is found in both the pigmented and nonpigmented layers. This type of picture predominates about the tip of the processes. In more basal areas the cells are cuboidal or columnar.

Transversal of these areas by electrodes often demonstrated differences in depth and potential delineation. Oblique insertions were common since the penetrations were about the apex of the processes. The various electrical alterations are illustrated in Figure 2.

A characteristic insertion is given in Figure 2-A. The nonpigmented epithelium is marked by a sudden negative downward deflection of 30 mv. With further penetration of the electrode, there again is a stepwise increase of 28 mv. With progression there is an incomplete return to the base line with a negativity of 2.0 to 3.0 mv. Upon withdrawal of the electrode, the cell delineations are again seen. The 30 mv. deflection was interpreted as penetration of the nonpigmented epithelium and the additional 28 mv. as entrance into the pigmented epithelium. The slight negativity was considered stroma.

Figure 2-A was the most characteristic picture; however, other potential determinations are illustrated in Figures 2-B and 2-C. In Figure 2-B the nonpigmented epithelium was well defined but was followed by a rapid insertion through the pigmented epithelium with a return to base line. In Figure 2-C no evidence of the nonpigmented epithelium was seen. Other variations of potentials were

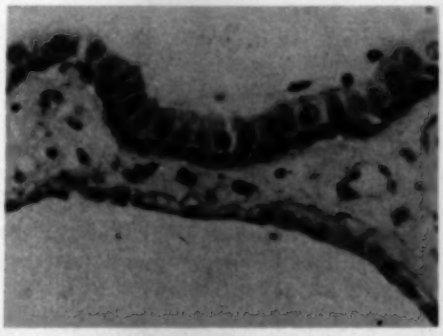

Fig. 1 (Miller and Constant). Histologic cross-section of rabbit ciliary process, showing variations in epithelium.

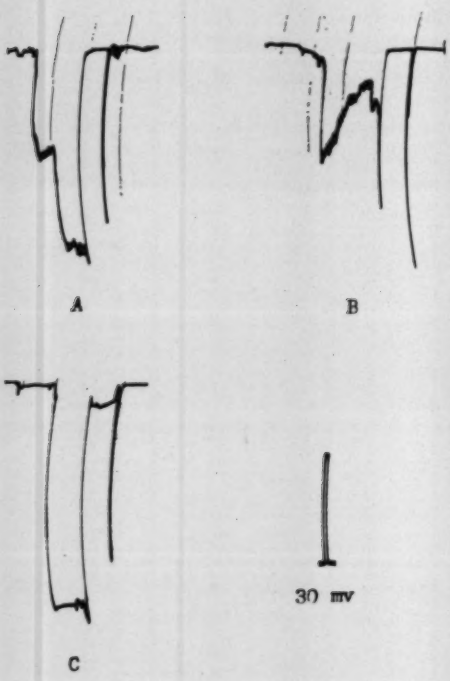

Fig. 2 (Miller and Constant). Various electrical alterations.

infrequent and were excluded from this study. Potassium cyanide was used to determine if the potential depended on active metabolism. Following the instillation of 100 mg. of potassium cyanide in the media, there was complete abolishment of all potentials after two minutes.

A. Normals

A total of 30 animals were used to determine normal potentials. The mean value of the nonpigmented epithelium was 27.8 mv. negative (standard deviation 3.1). The pigmented epithelium averaged 55.6 mv., with a standard deviation of 4.4. The stroma was determined at 5.1 mv. negative (standard deviation 1.8). (See Table 1).

Initially a group of animals were enucleated by killing with intracardiac air injection or while under sodium phenobarbital anesthesia. In both situations the results were identical so that it was felt that barbiturate anesthesia did not alter the potential. The first 18 animals of the 30 normals were determined while oxygen was forced through the media. The remaining 12 were done without oxygenation. There was no distinguishable difference between the two groups at the end of 45 minutes.

The depth of penetration was determined as well as the potential variation. The average distance between surface and pigmented epithelium was 25 microns, with a standard deviation of 12. The pigmented epithelium measured 29 microns, with a standard deviation of 17. The variation between measurements was thought to be due to the angle of penetration; in some areas it was oblique, in other areas perpendicular.

B. The effect of pH

The influence of pH variations was determined in eight eyes. No alteration in potential was noted with a pH of 7.0 to 7.6. With a pH of 6.8 there was a decline in potential that began at the end of 30 minutes. At the end of one hour the values for the two layers of epithelium were two-thirds of their original determinations. Below pH 6.5 a marked depression was seen within 12 minutes, with all total measurements of 15 mv. or less. Concomitant with this, the lens became cataractous. Similar results have been noted with alkaline media but the exact pH measurements have not been determined.

C. Carbonic anhydrase inhibitors

Five anesthetized animals were used for acetazolamide determinations. The first eye was enucleated and potential values determined. Ten mg. of acetazolamide was placed in the media and the procedure repeated. No difference was noted from the control and the in vitro determinations (Table 1).

100 mg. per kg. of acetazolamide was administered intravenously, and the second eye determined at 20 to 30 minutes. A depression of potential was seen in the nonpigmented epithelium. However, the standard deviation was of sufficient magnitude that the results

were inconclusive. Similar results were obtained with a 20 mg./kg. intravenous dosage of methazolamide in 11 animals. The second eye was enucleated from five to 30 minutes following the injection.

D. Glucose

Glucose was administered to seven animals in a dosage of 0.25 to 0.50 gram per kg. intravenously. Following a 30 minute interval, a depression of both cells was found in the experimental eye. The control eye measured 27.8 mv. for the nonpigmented epithelium (standard deviation 4.9); and the pigmented epithelium was 58.7 mv. (standard deviation 5.0). The stroma was 5.5 mv. (standard deviation 2.1). After the glucose the nonpigmented epithelium was decreased to 23.5 mv. (standard deviation 4.2); and the pigmented epithelium 48 mv. (standard deviation 4.5). The stroma remained at 5.4 mv. (standard deviation 1.9).

As a control to determine the effect of an intravenous administration of a sugar, sucrose was substituted. This was given in a dosage of 0.50 gram per kg. and the experiments repeated in five animals. No alteration was noted following the administration of this nonmetabolized sugar. In four additional animals one gram per kg. was used with similarly negative results.

It did not appear that glucose in the media was required for the maintenance of potentials in vitro, since experiments in four animals failed to show any change between the control eye in Tyrode's Solution with glucose and the second eye in glucose-free media for a 30 minute incubation period.

E. Insulin

During the determination of normal values it was noted that the addition to the media of 0.1 to 0.3 units of regular insulin per cc. would elevate the potential values, provided they were subnormal initially. Similarly, the depressed potential values following intravenous glucose were elevated by in vitro insulin to approximately normal determinations. Simultaneous intravenous insulin with glucose prevented the potential change seen after glucose alone. Insulin did not have any influence upon the depression resulting from acid media.

F. Starvation

During the preliminary determinations it was felt that starvation did increase potentials. This observation was not verified with the development of a better technique and more constant electrodes.

In five animals an increase of 1.8 mv. was noted in the nonpigmented epithelium and 4.3 mv. in the pigmented epithelium. The standard deviation is of sufficient magnitude that the results were inconclusive.

Discussion

The present studies were initiated in the hope that the maintenance and alteration of potential would relate to the secretory mechanism of the ciliary body. However, under the present experimental conditions the average potentials found five to 30 minutes (time of maximal vacuole formation ultramicroscopically) after systemic administration of carbonic anhydrase inhibitors did not differ significantly from the control eye. The variation of cell type and the variation in distribution of potentials of cells over the ciliary process made detection of small potential changes difficult, even under the controlled conditions of these experiments.

Although the inhibition of secretion by carbonic anhydrase inhibitors alters the composition of aqueous, perhaps by affecting the "sodium pump" and/or intracellular pH, the net magnitude of these effects on the electrolyte composition of the cells of the ciliary epithelium and, consequently, their predicted effect on intracellular potentials, have not been determined. In a preliminary attempt to assess such changes indirectly, ciliary bodies were incubated with strophanthidin, but decreases in potentials of ciliary epithelial cells were not found until 30 to 50 minutes after the introduction of the in-

hibitor of the "sodium pump" into the media. Whether one might disturb sufficiently the electrolyte balance maintained in vivo to detect more uniform potential changes of greater magnitude by prolonged administration of acetazolamide has not as yet been studied. The nonuniform distribution of cellular potentials over the ciliary processes indicate, if potential is related to secretion, a variation in secretory activity between cells of some magnitude. The technical difficulties in subjecting the ciliary body to flux measurements and analytically determining electrolyte changes in these cells of variable potentials present an interesting challenge. It is hoped that the combined use of tonography and potential measurements in selected animals of high or low secretion will aid in establishing the relationship of secretory and potential changes.

As shown by the work of Zierler[7] the electrolyte (K^+) change predicted on the basis of observed potential change may not occur. In agreement with the findings of Zierler who was studying muscle, insulin was found to increase rapidly the polarization of the membranes of the epithelial cells. This effect was especially apparent in instances where a decreased negative potential was found in untreated eyes, or after the intravenous administration of glucose.

In contrast to muscle, a hyperpolarization effect was not seen within the variability of the present procedure. Injection procedures may stimulate adrenalin release which may alter the permeability of membranes (Lundberg[8] and Kipnis[9]). This in vivo effect was controlled by the use of sucrose injection which did not result in a change in potential of ciliary body epithelial cells. Our preliminary studies indicate that the hyperglycemic diabetic rabbit and the normal rabbit injected with glucose may have similarly decreased negative potentials. The data at present are too few and the relationships too complex to enable one to draw conclusions as to the effect of insulin on the potential of the ciliary body epithelial cells. Further studies are in progress on the effects of inhibitors and metabolic disturbances in vivo and in vitro on these potentials.

Summary

1. A potential gradient of 28 millivolts was found between the pigmented and nonpigmented epithelium of the rabbit.

2. The nonpigmented epithelium averaged 27.8 mv. and the pigmented 55.6 mv. negative.

3. No conclusive alteration was observed after acetazolamide in vivo or vitro.

4. Glucose in vivo depressed potentials.

5. Insulin elevated decreased values that occurred spontaneously or from glucose.

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Discussion

Albert M. Potts, M.D. (*Chicago*): The principle on which this work was started is a sound one and one which has produced useful results in allied areas in the past. Wherever an ionic concentration difference exists across a biologic membrane, one may expect to detect an electrical potential difference if two suitable electrodes are placed one on either side of the membrane. If the membrane in question is a single small cell, the size of the connection to one of the electrodes must be much smaller than the size of the cell wall; thus the less than one micron tip of the glass pipette used. On the basis of previous experience such a concentration difference and corresponding potential difference may be expected across every living cell and comprises the so-called "resting potential."

The mechanism by which the concentration difference is obtained is a complex one involving static phenomena such as the Donnan membrane equilibrium and membrane charge, as well as active phenomena such as active transport of sodium ion. The details of this mechanism are still being elucidated. Certain specific cells such as the acid secreting cells of the stomach have an additional function which involves active transport of ions other than sodium and the operation of such a mechanism should affect the final value of the measured potentials. Further, one might expect the value of the potential to fluctuate with secretory activity of the cell and that the value of the potential might be a measure of this secretory activity. It was this expectation which motivated this research on the ciliary body.

What they found was the expected resting potential which showed three changes corresponding to the three boundaries of the two cells of the ciliary epithelium. This corresponds exactly to the findings of Berggren and represents independent confirmation of the ciliary resting potentials. Then Miller and Constant wished to go farther and isolate the potential connected with the special secretory activity of the ciliary body and for this purpose used amounts of Diamox known to sharply inhibit secretion of aqueous. However, measurements on ciliary bodies removed from animals so treated showed no statically significant difference from those of untreated animals. A number of possible explanations for this negative result suggest themselves. Absence of secretion in aqueous formation, or too small an ion change to be measurable, seem highly unlikely on the basis of available knowledge. More likely is the possibility that an intact organoid with functioning circulation is necessary for estimation of secretory activity. Some hint of this is found in the large difference in the second potential step between the in vitro and in vivo experiments of Berggren. Thus, if the in vivo experiment with ciliary body successfully transplanted to the anterior chamber could be repeated before and after Diamox, the expected potential change might be found. Since this type of measurement might be used to follow and interpret many types of pharmacologic effects on aqueous formation, it is highly desirable that the authors continue this promising line of research.

Dr. Monte G. Holland (New Orleans): I would like to report on some experimental work that we have done in our laboratory along similar lines.

We have followed a technique that is somewhat different from that of Dr. Miller. We have removed the ciliary body in toto from cat eyes and mounted the preparation as a membrane between two reservoirs, similar to the experiments Dr. Potts has reported on the cornea. We have been able to measure the membrane potential of the isolated ciliary body of the cat.

We found that the membrane potential of the preparation treated in this fashion was somewhat lower; it was about 1.2 to 2 millivolts, with the stromal side of the preparation positive. This magnitude of potential is somewhat similar to that Dr. Potts has found with the cornea.

We have also done preliminary experiments with enzyme inhibitors and have found that cyanide abolishes the potential. We have also observed an effect with Diamox. We exchange the fluid with an identical fluid but containing 0.2 percent Diamox and there is a rapid decline of the membrane potential to approximately half of its initial value, followed by a much slower decline.

These enzyme inhibitor studies are still under investigation, and we have submitted a publication on this work. I should like to compliment Dr. Miller on a very interesting study.

Dr. Ludwig von Sallmann (Bethesda, Maryland): I would like to ask one question. Neither in the presentation by Dr. Miller nor in the paper by Berggren mention is made of possible resistance changes in the microelectrode during this procedure.

I was told recently by Dr. Fuortes that in introducing the microelectrode into the tissue small bits of it often block the opening of the microelectrode and may give rise to a faulty reading by increasing the resistance of the electrode. I wonder whether the authors have had any experience with this.

Dr. James E. Miller (closing): I would like to thank both Dr. Potts and Dr. Holland for their comments. Dr. Holland, I am sure, is aware of the fact that he is dealing with a rather complex mem-

brane that is made up of many cells, and is in turn measuring a gradient on both sides of this membrane. We were measuring intracellular potentials; so, one would tend to expect a large difference between these two experiments.

Concerning Dr. von Sallmann's comment, we did actually measure the resistance of this electrode throughout the experiment. This is described in the paper. This method was also acquired from the National Institutes of Health, since we corresponded quite freely with Dr. K. Frank and also Mrs. Becker in his laboratory. The technique that was used is one that was described by Dr. Frank and Dr. Fuortes.

THE TRANSPORT OF ORGANIC ANIONS BY THE RABBIT EYE

I. In vitro iodopyracet (diodrast) accumulation by ciliary body-iris preparations*

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The failure of a number of organic acids (such as penicillin, paraaminohippurate, phenolsulfonphthalein, iodopyracet)to gain access to the posterior chamber or vitreous of the eye raises questions as to the nature of the barrier and its role in the formation and regulation of ocular fluids.[1,2,3] Many of these same organic acids are known to be accumulated by renal slices in vitro and to be rapidly secreted into the renal tubular fluid by the kidneys of all vertebrate species studied.[4] The present series of studies demonstrate that these acids are accumulated actively by the excised rabbit ciliary body and are secreted out of the eye in the living animal. The results of the in vitro and in vivo studies closely parallel the classic experiments on renal tubular transport of the same compounds.

Method

Albino rabbits weighing approximately 2.0 kg. were killed by air embolus. Their eyes were promptly enucleated and opened posteriorly so as to expose the lens and ciliary body. The lens and vitreous were carefully dissected free. The ciliary body and iris were removed as a complete ring. It was used as such for some experiments; in others it was cut in half.

The ciliary body-iris preparation was incubated in one ml. of Tyrode's solution containing 10 μg./ml. (2.5×10^{-5} M) iodopyracet (Diodrast) labeled with I^{131} (approximately 0.05 to 0.2 μc./ml. Tyrodes). The Tyrodes used had the following composition (per liter):

Sodium chloride	8.00 gm.
Potassium chloride	0.20 gm.
Calcium chloride	0.20 gm.
Magnesium chloride	0.10 gm.
Sodium phosphate	0.05 gm.
Sodium bicarbonate	1.0 gm.
Glucose	1.0 gm.

The media were adjusted to pH of 7.45 to 7.50 by bubbling CO_2 through the solution. Before use the Tyrode's solution was oxygenated for five to 10 minutes.

The Diodrast^{131} was the commercial preparation supplied by Abbott Laboratories. It has been demonstrated that the I^{131} remains securely affixed to the Diodrast.[5] Incubations were carried out with gentle shaking in a water bath at 37°C for 60 minutes. In addition, time series were run as well as incubations at other temperatures. At the end of the incubation the ciliary bodies were blotted, weighed and counted in a well type scintillation counter. Counts were compared with measured amounts of the incubation fluid. Weighing of the ciliary body iris preparations after drying to constant weight revealed that approximately 85 percent of the

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ciliary bodies were water. All values presented are corrected to a tissue water basis.

Results

1. Accumulation

After one hour's incubation there were approximately 10 to 15 times as many counts per unit volume in the tissue water as in the incubation media. Although ciliary body-iris preparations from different animals varied from a ratio of 5.0 to 20 (mean of 12.2 for 250 animals) under similar conditions of incubation, the two eyes of the same animal or two halves of the same eye provided values in very close agreement. Therefore, all studies on requirements, inhibitors, temperature effects and so forth were done by comparing specimens from the same animal.

2. Requirements in the media

A marked decrease in accumulation was noted when the medias were made potassium free or glucose free. The absence of calcium from the media had no significant effect nor did the addition of 0.01 molar acetate, lactate, pyruvate, mannose, galactose, citrate, or malate. Of special interest in terms of the composition of ocular fluids was the finding that additions of various concentrations of urea, ascorbate or hyaluronate had little or no effect on accumulation of iodopyracet.

The glucose in the media (100 mg. percent) could not be replaced by lactate, pyruvate, acetate, succinate, citrate or galactose. Fructose and mannose did not restore activity to the glucose free media when used at 100 mg. percent concentrations but did replace glucose successfully when concentrations of the two sugars were increased to 500 mg. percent. Variations of pH between 7.2 and 8.5 had little effect on the concentrating ability of the tissue. Outside of this range a progressive decline in activity was noted.

No accumulation occurred in the absence of oxygen (for example, bubbling nitrogen through the media).

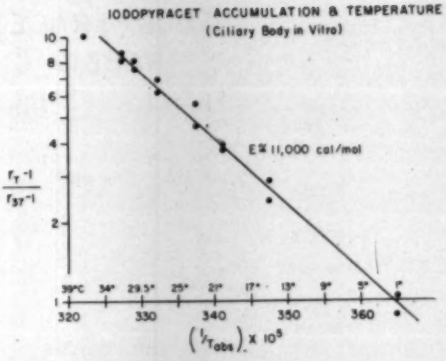

Fig. 1 (Becker). The effects of variation of the temperature of incubation on the accumulation of iodopyracet (Diodrast) by the rabbit ciliary body-iris preparation in vitro. r_T = ratio of concentration in ciliary body to that in media at temperature T; r_{37} = ratio at 37°C.

3. Temperature dependence

The accumulation of labeled Diodrast in the ciliary body-iris preparation was completely inhibited at 0°C. Activity increased with temperature as demonstrated in Figure 1 in such fashion that it more than doubled for every 10°C rise in temperature up to 34°C ($Q_{10} = 2.1$). An Arrhenius type plot revealed a linear relationship between log accumulation (as a fraction of the 37°C value) and the reciprocal of the absolute temperature of incubation. The slope of the line suggested an activation energy of approximately 11,000 cal./mol. At temperatures above 40°C, accumulation decreased and at 55°C no accumulation occurred.

4. Variation of substrate concentration

With increase in concentration of non-labeled iodopyracet in the media, the ratio of the radioactivity of tissue to media decreased progressively to unity. This corresponded to an increase in the amount of Diodrast accumulated by the tissue up to a maximum value of about 10 to 12 μg/ciliary body/hour (0.5 − 0.6 mM/kg./hour). The data obtained for the four half ciliary body-iris preparations of individual animals could

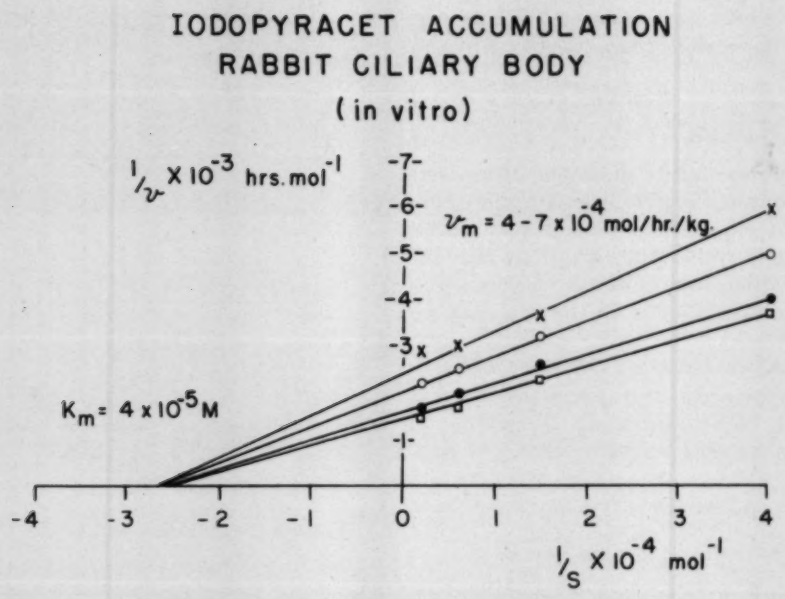

Fig. 2 (Becker). Lineweaver-Burk plot of the accumulation of iodopyracet in vitro by the ciliary bodies of four different animals. Each point represents the reciprocal of the accumulation for one-half ciliary body-iris preparation plotted against the reciprocal of the substrate concentration in the media. s = substrate concentration; v = accumulation in tissue at one hour (37°C); K_m = apparent Michaelis-Menten constant; v_m = maximum rate of accumulation at one hour.

be fit to Lineweaver Burk type plots (fig. 2). These revealed linear relationships between the reciprocal of accumulation per hour and reciprocal of substrate concentration. Such plots resemble Michaelis-Menten type saturation kinetics.[6,7,8] The apparent Michaelis-Menten constant, K_m (half-saturation concentration) approximated 4×10^{-5} M in most animals, and the maximum velocity was estimated at $4 - 7 \times 10^{-4}$ M/hour/kg./tissue. This would correspond to an average rate of approximately 5×10^{-10} M/min./ciliary body. If shorter incubation times are used, the maximum velocity approaches approximately 2×10^{-9} M/min./ciliary body.

5. Inhibitors[9,10,11,12,13,14]

A variety of metabolic inhibitors were demonstrated to abolish the capacity of the tissue to concentrate Diodrast when added to the media. These included dinitrophenol, iodoacetate, fluoride, cyanide, fluoroacetate, phlorizin, methylene blue and malonate. In Table 1 are presented concentrations of these agents in the incubation fluid that resulted in 50 percent inhibition. These values were estimated from linear plots of the reciprocal of the accumulation vs. inhibitor concentra-

TABLE 1

EFFECTS OF METABOLIC INHIBITORS ON IN VITRO ACCUMULATION OF IODOPYRACET (DIODRAST) IN RABBIT CILIARY BODY-IRIS PREPARATIONS

Inhibitor	Apparent K_i* (50% inhibition)
Dinitrophenol	1×10^{-5} M
Cyanide	2×10^{-4} M
Iodoacetate	1×10^{-5} M
Fluoroacetate	5×10^{-4} M
Fluoride	1×10^{-4} M
Malonate	5×10^{-3} M
Methylene blue	4×10^{-5} M
Phlorizin	1.5×10^{-4} M

* Determined from the linear plots of the reciprocal of the amount accumulated against the concentration of inhibitor in the media (average values of four to 10 rabbits).

tion. All results listed are mean values for four to 10 rabbits.

The addition to the media of a number of organic acids that are believed to compete for this transport system in the kidney were also found to reduce the accumulation of Diodrast in the ciliary processes. These included paraaminophippurate (PAH), phenolsulfonphthalein (PSP), penicillin, urate, probenecid, benzmalacene, salicylate, chlorothiazide, mercuhydrin, caprylic acid, succinate, bromcresylgreen, phenolphthalein glucuronide and menthol glucuronide (Table 2). There is sufficient scatter in the data so that it is not possible to prove by Lineweaver Burk type plots that these agents are competitive inhibitors.

Interestingly enough caproic acid was found to inhibit at concentrations about 10^{-3}M but to potentiate accumulation at concentrations of 10^{-4}M.

The systemic administration of Benemid to the rabbit (250 mg./kg. intraperitoneally) 60 to 90 minutes before removing the eye resulted in 80 to 90 percent inhibition of subsequent accumulation in vitro. However, no effects could be obtained by the systemic administration of penicillin, dinitrophenol, Diodrast, Diamox or ascorbate.

TABLE 2

EFFECTS OF "COMPETITIVE" INHIBITORS ON IN VITRO ACCUMULATION OF IODOPYRACET (DIODRAST) IN RABBIT CILIARY BODY-IRIS PREPARATIONS

Inhibitor	Apparent K_i* (50% inhibition)
Hexanoate	1×10^{-3} M
Caprylate	5×10^{-4} M
Succinate	5×10^{-4} M
Urate	1×10^{-3} M
Acetazolamide	4×10^{-4} M
Chlorothiazide	5×10^{-4} M
Mercuhydrin	1×10^{-5} M
Salicylate	1.5×10^{-3} M
Bromcresylgreen	7×10^{-7} M
Phenolphthalein glucuronide	1×10^{-4} M
Menthol glucuronide	1×10^{-4} M
Penicillin	1.3×10^{-4} M
Probenecid	1×10^{-4} M
Benzmalacene	2×10^{-4} M
Para-aminohippurate (PAH)	2×10^{-4} M
Phenolsulfonphthalein (PSP)	3×10^{-4} M

* Determined from the linear plots of the reciprocal of the amount accumulated against the concentration of inhibitor in the media (average values of four to 10 rabbits).

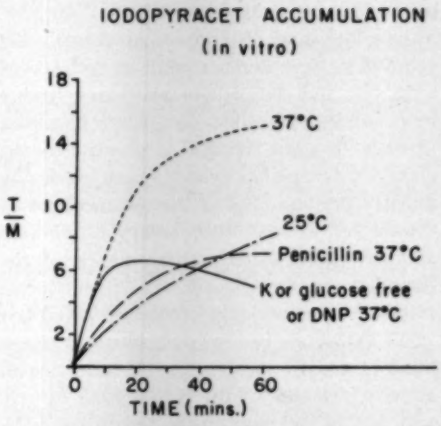

Fig. 3 (Becker). The time course of Diodrast accumulation in vitro by the rabbit ciliary body-iris preparation. Note the reduced rate when temperature is lowered from 37°C to 25°C. Omitting potassium or glucose from the media or adding such metabolic inhibitors as dinitrophenol also decrease accumulation. The penicillin curve demonstrates the effects of a competitive inhibitor at a concentration resulting in approximately 50 percent inhibition. $\frac{T}{M}$ = ratio of concentration in tissue water to that in media.

In series in which samples of media were removed at various time intervals of incubation, one could demonstrate the rate of approach to the one hour level at 37°C (fig. 3). In the absence from the media of potassium or glucose or on the addition of dinitrophenol, it is apparent that accumulation is markedly impaired after the first few minutes. This may be compared with the reduced capacity for accumulation of Diodrast in the presence of suitable concentrations of penicillin. At lower temperatures (for example, 25°C) the rate of accumulation is decreased.

DISCUSSION

On the basis of the ability of the ciliary body-iris preparations to accumulate iodopyracet against a concentration gradient, the temperature dependence of the reaction, the

need for oxygen, certain ions, suitable metabolites and energetic processes, the saturation type kinetics and the effects of metabolic and competitive inhibitors, it may be postulated that this is an active transport process. In this regard is resembles the ability to accumulate related organic acids by in vitro preparations of the metanephros of the chick,[15] rat and rabbit embryonic choroid plexus,[16] the isolated renal tubules of the flounder or frog,[17] and the rabbit renal tubule.[18] The major differences noted are small variations in the concentrations of inhibitors needed for comparable reduction of activity, the lack of the potentiation by acetate, lactate and pyruvate in the ciliary body, as compared to its effect in the kidney and the dependence of the ciliary body-iris preparation on glucose in the media.

It should be emphasized that the accumulation ratios of 10 to 15 were for ciliary body-iris preparations. From histologic studies using phenol red or chlorphenol red and from crude dissections of ciliary body-iris preparations, it is evident that almost all of the accumulated anions are in the ciliary processes. Since the processes are less than 20 percent by weight of the whole preparation, the concentrations attained in the ciliary processes must be 50 to 75 or more times that of the media.

The findings by Friedenwald of the transfer of various anionic dyes to the stroma of the rabbit ciliary body appears to be closely related to the present study.[19] This accumulation was also blocked by cyanide or anoxia. Friedenwald believed this to demonstrate and depend upon an electron transport mechanism which was intimately connected with the formation of aqueous humor. The present work confirms the fact that the ciliary body can accumulate organic anions and that it is an active process requiring metabolic energy. However, there is little evidence at present that this transport system is intimately associated with the rate of aqueous flow or the maintenance of intraocular pressure. In fact, it can be demonstrated that the Diodrast transport system can be inhibited in vivo by such agents as Benemid without altering intraocular pressure or rate of aqueous secretion.

Summary

Freshly excised preparations of rabbit ciliary body and iris accumulate trace amounts of I^{131} labeled iodopyracet to ten to fifteen times the concentration in the incubation media (Tyrode's solution). The ability to concentrate iodopyracet requires glucose, oxygen and potassium and is temperature dependent ($Q_{10} = 2.1$). It is inhibited by cyanide, fluoride, iodoacetate, malonate phlorizin, methylene blue, dinitrophenol and fluoroacetate. The concentrating mechanism demonstrates saturation kinetics and is also inhibited by the presence of other organic anions such as penicillin, paraaminophippurate, phenolsulfonphthalein, probenecid, Benzmalacene, salicylate, glucuronides and Bromcresylgreen.

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ACKNOWLEDGEMENT

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THE TRANSPORT OF ORGANIC ANIONS BY THE RABBIT EYE

II. IN VIVO TRANSPORT OF IODOPYRACET (DIODRAST)*

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A barrier has been demonstrated which restricts the penetration of iodopyracet and such related organic anions as penicillin and paraaminohippurate from the plasma into the intraocular fluids of the rabbit.[1, 2] This paper reports an investigation of the movement of iodopyracet across this barrier in the opposite direction, that is, from intraocular fluids to plasma. The methodology is that described by Maurice in the study of sodium turnover in the vitreous.[3] The results establish the existence of an active transport process out of the eye for these anions.

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† From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine.

METHODS

I. TRACE DOSE EXPERIMENTS

A solution of I^{131} labeled iodopyracet prepared by Abbott Laboratories (Radio-Diodrast Sterile Solution) containing 5 to 10 mg. per ml. iodopyracet with an initial specific activity of approximately 100 μc./mg. was used as the radioactive tracer. A volume ranging between 2.0 and 15 μl. (0.02-0.15 mg. iodopyracet) adjusted to contain approximately 1.0 μc. was drawn up into a number 705 Hamilton Microliter Syringe. One eye of an unanesthetized albino rabbit weighing approximately 2.0 kg. was proptosed, and the needle of the microliter syringe was inserted through the sclera approximately 1-2 mm. posterior to the equator and guided under direct visualization through the pupil into the center of the vitreous. Care was taken to avoid penetration of

the lens. The solution was slowly injected into the vitreous and the needle withdrawn concurrent with release of external pressure on the eye. Initially, topical 0.5 percent pontocaine was used prior to proptosis but in the later experiments no local anesthetic was employed. The amount of iodopyracet remaining in the eye at various times after injection was determined by means of an external counting system. A scintillation well counter (model DS-3 Nuclear-Chicago) turned on its side was used as the external probe, with the injected eye maintained at a fixed distance from the counting chamber. The initial counting rate was 50-100 × 256 CPM under these conditions, with a background of 3.5-4.0 × 256 CPM. Following completion of the external counting, the injected eye was again proptosed and anterior chamber and vitreous taps were performed. These samples were assayed within the well of the same scintillation counter with a background of 1.5-2.0 × 256 CPM.

II. Saturation dose experiments

Solutions of 10-70 percent nonlabeled iodopyracet were mixed in varying proportions with the tracer solution. Between 3 and 25 µl. of the resulting solutions providing total doses of 0.3-14.0 mg. iodopyracet were used for intravitreal injections. Counting and tapping were performed in the same manner as in the trace dose experiments.

III. Inhibition studies

In addition to intravitreal injection of trace or saturating doses of iodopyracet with subsequent external counting and tapping, these rabbits also received effective inhibitory agents.

A. *Systemic inhibition.* An intraperitoneal injection of 150-200 mg./kg. of probenecid was administered prior to the intravitreal injection and was repeated at four hour intervals throughout the duration of the experiment.

B. *Intravitreal inhibition.* Solutions of penicillin or paraaminohippurate were mixed in varying proportions with the tracer solution of iodopyracet and the resulting solutions were used for the intravitreal injections.

IV. Penetration studies

Approximately 50-100 µc. of I^{131} iodopyracet was administered intravenously to six albino rabbits immediately following bilateral nephrectomy performed under ether anesthesia. Three of these animals received 0.6-1.4 gm. nonlabeled iodopyracet intravenously and one of them received an intravitreal injection of 15 µl. of normal saline in one eye (check on possible role of nondiffusible plasma binding sites and effect of prior vitreous injection). Taps of anterior chamber, vitreous and blood were performed at intervals of six to 24 hours following the intravenous injection. The samples of aqueous, vitreous and plasma were then assayed in the scintillation counter.

V. Uphill gradient studies

Bilateral nephrectomy was performed under surital anesthesia on five albino rabbits. The following morning three animals received 1.0 gm. of nonlabeled iodopyracet intravenously. After 30 minutes all five animals were given unilateral intravitreal injections of trace doses of labeled iodopyracet. Periodic external counting over the injected eyes was performed in the usual manner.

Results

I. Trace dose experiments

The loss of a trace dose of intravitreal iodopyracet from the eye followed a single exponential curve as shown for a representative animal in Figure 1. The turnover rates for the first 20 rabbits ranged from a half life of 140 minutes to 230 minutes with a mean value of 177 minutes. All taps performed on these animals revealed zero concentration of iodopyracet in the anterior chamber. It is important to note that all of the iodopyracet leaving the eye could be very rapidly recovered quantitatively in the urine. Since there was no significant accumulation

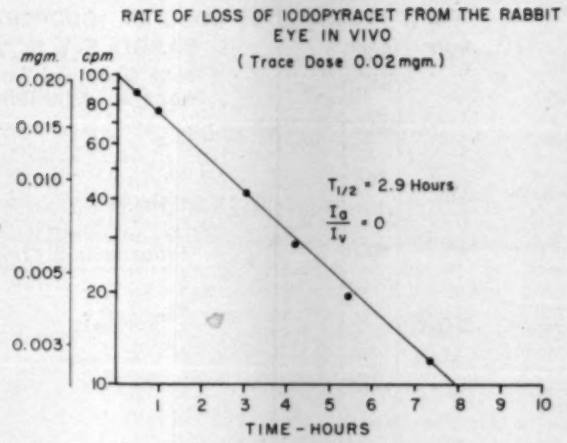

Fig. 1 (Forbes and Becker). Representative example of rate of loss of intravitreal trace dose (0.02 mgm.) of I^{131}-iodopyracet measured with external counter (semilog plot). Ordinate I_v = amount of iodopyracet in the vitreous expressed as counts per minute with corresponding milligram values. Single exponential curve with half-life 2.9 hours. No iodopyracet in the anterior chamber.

in the body, the external counting rate corresponded accurately with the amount of iodopyracet in the eye.

Following this type of intravitreal injection, slitlamp and fundus examination were within normal limits. Furthermore, tonography and anterior chamber chemistries (that is, bicarbonate, chloride, and ascorbate) did not differ significantly from the opposite noninjected eye.

II. Saturation dose experiments

As the amount of nonlabeled iodopyracet combined with the trace dose was increased, the semilogarithmic plots of activity versus time became curvilinear with progressively decreasing slopes approaching a half life of 17 hours. Concurrent with the slower loss from the eye, iodopyracet gained access to the anterior chamber in increasing quantities so that the ratio (r) of the amount in the anterior chamber (I_a) to that in the vitreous (I_v) approached a limiting value of 0.04. For sufficiently large doses these limiting values were nearly attained and the process once again became a single exponential but with much longer half life (fig. 2).

III. Inhibition studies

A. *Systemic inhibition.* Following intraperitoneal administration of probenecid the rate of loss of the trace dose of iodopyracet was markedly diminished from the three hour half life to one of 17 hours. During the phase of reduced rate of exit, iodopyracet entered the anterior chamber (fig. 3). Animals receiving probenecid plus a saturating dose of iodopyracet in the vitreous also followed a single exponential curve with half life of 17 hours and with sufficient time the ratio, r, approximated 0.04.

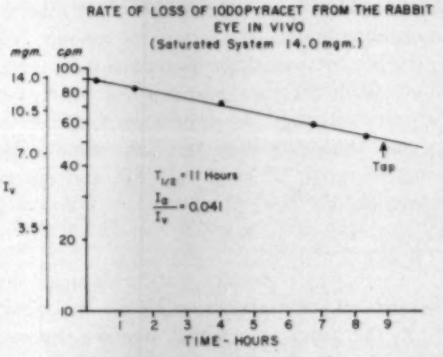

Fig. 2 (Forbes and Becker). Representative example of rate of loss of intravitreal saturating dose of I^{131}-iodopyracet (14.0 mg.) measured with external counter (semilog plot). Ordinate I_v = amount of iodopyracet in the vitreous expressed as counts per minute with corresponding milligram values. Slope corresponds to 11.0 hour half-life turnover rate. I_a/I_v, ratio of amount of iodopyracet in the anterior chamber to amount in the vitreous equals 0.041. Turnover rate and anterior chamber accumulation ratio approach limiting values for saturated system.

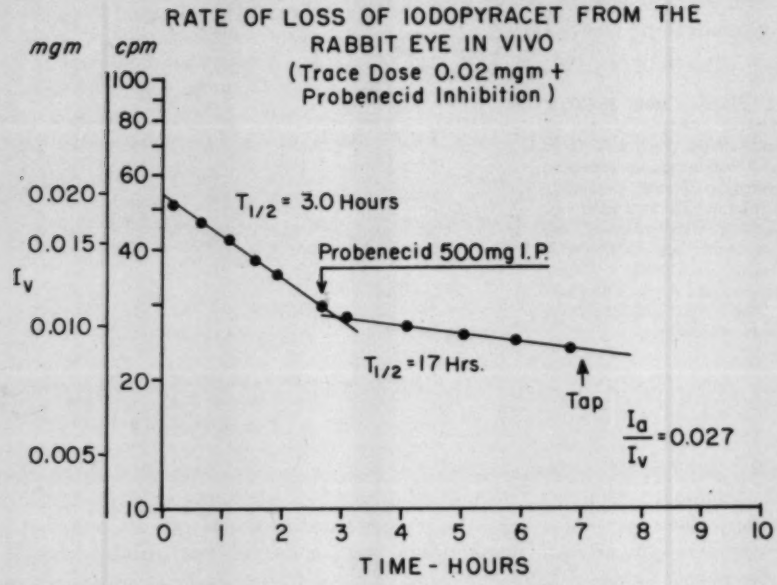

Fig. 3 (Forbes and Becker). Representative example of diminished rate of loss of intravitreal trace dose (0.02 mg.) of I^{131}-iodopyracet following 500 mg. probenecid intraperitoneally (semilog plot). Turnover rate rapidly shifts from 3.0 hour half-life to 17.0 hour half-life, the limiting value. $I_a/I_v = 0.027$ after 4 hours of inhibition.

B. *Intravitreal inhibition.* The results of these studies were very similar to the saturation experiments. As increasing amounts of penicillin or paraaminohippurate were combined with the trace dose of iodopyracet, the semilogarithmic plots developed progressively decreasing slopes with the same limiting value of 17 hour half life and corresponding r = 0.04 (Fig. 4).

IV. Penetration studies

There was a definite restriction upon the movement of iodopyracet from the blood into the anterior chamber aqueous humor. This was previously demonstrated by Barany and Kinsey[1] who reported an average steady state distribution ratio of 0.14 for concentration in aqueous to that in plasma. The present results are essentially in agreement with this value. The vitreous humor, however, was found to be virtually impenetrable to plasma iodopyracet with steady state distribution ratios for concentration in vitreous to that in plasma ranging from 0.00 to 0.02. The systemic administration of probenecid to

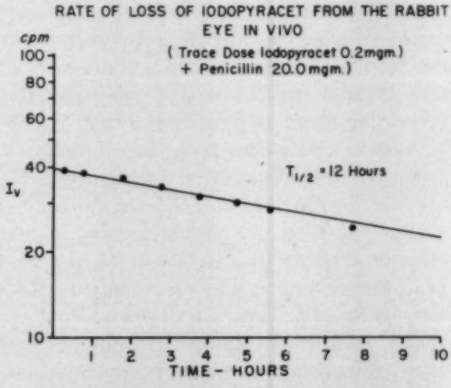

Fig. 4 (Forbes and Becker). Representative example of diminished rate of loss of intravitreal trace dose (0.20 mg.) of I^{131}-iodopyracet produced by 20.0 mg. of penicillin in the vitreous (semilog plot). Slope corresponds to 12.0 hour half-life turnover rate, approaching the limiting value for saturated system.

TABLE 1

IODOPYRACET TRANSPORT AGAINST A CONCENTRATION GRADIENT IN NEPHRECTOMIZED RABBITS

Rabbit No.	Intravitreal Iodopyracet (Tracer Solution)	Intravenous Iodopyracet (70% Solution)	Half-Life (Mins.)
1	4×10^{-5} gm.	1.0 gm.	185
2	4×10^{-5} gm.	1.0 gm.	160
3	4×10^{-5} gm.	1.0 gm.	180
4	4×10^{-5} gm.	0	180
5	4×10^{-5} gm.	0	205

nephrectomized animals failed to increase penetration into the vitreous or aqueous humors.

V. Uphill gradient studies

All five nephrectomized animals followed the usual single exponential curve for a trace dose. There was no significant difference between the turnover rates of the nephrectomized and intact rabbits. Furthermore, the rate of secretion out was not altered by the high plasma iodopyracet level resulting from one gram of nonlabeled iodopyracet given intravenously (Table 1).

Discussion

The major criteria required for postulating an active transport process[4,5] have been established in connection with the movement of iodopyracet out of the rabbit eye, including saturation phenomena, effects of inhibitors and transport against a concentration gradient.

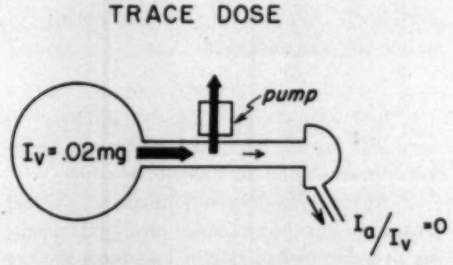

Fig. 5 (Forbes and Becker). Schematic representation of mechanism of loss of intravitreal trace dose of iodopyracet from the rabbit eye in vivo. Rapid secretion out of the eye by the transport system (pump) prevents access to the anterior chamber.

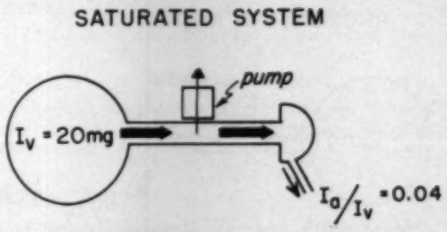

Fig. 6 (Forbes and Becker). Schematic representation of mechanism of loss of intravitreal saturating dose (20.0 mg.) of iodopyracet from the rabbit eye in vivo. The contribution of the transport system operating at maximum capacity is relatively insignificant. Almost all of the iodopyracet reaches the anterior chamber where it accumulates to ratio $I_a/I_v = 0.04$, and leaves the eye by flow and diffusion.

A. Saturation phenomena

It has been demonstrated that the rate of loss is not a linear function of the concentration within the eye. The turnover rate of 0.004 min.$^{-1}$ ($T\frac{1}{2}$ approximately three hours) for trace doses is accomplished posteriorly by an active secretory process which is sufficiently rapid to prevent diffusion into the anterior chamber (Fig. 5). With progressively increasing concentrations of iodopyracet, the maximum transport capacity is exceeded, producing a progressive decline in turnover rate and allowing accumulation in the anterior chamber. The limiting turnover rate of 0.0007 min.$^{-1}$ ($T\frac{1}{2}$ approximately 17 hours) and ratio $r = 0.04$ correspond to exit from the anterior chamber by flow and diffusion mechanisms which are independent of concentration. At these high saturation levels the maximum contribution of the transport system is negligible (Fig. 6).

B. Effect of inhibitors

The administration of other suitable organic anions has been shown to block the secretion of trace levels of iodopyracet, thereby permitting anterior chamber accumulation and consequent loss through the flow-

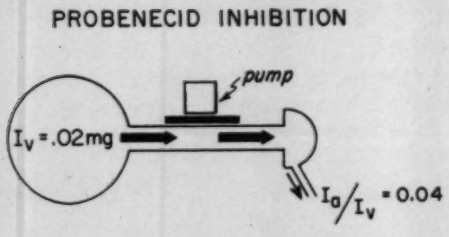

Fig. 7 (Forber and Becker). Schematic representation of mechanism of loss of intravitreal trace dose of iodopyracet under probenecid inhibition. With complete inhibition of transport system all iodopyracet reaches the anterior chamber and leaves the eye by flow and diffusion. Accumulation ratio $I_a/I_v = 0.04$.

diffusion mechanism (Fig. 7). These substances may be considered as inhibitors which compete for the active transport sites rather than acting on energy-supplying mechanisms.[5]

C. Transport against an uphill concentration gradient

The systemic administration of large doses of iodopyracet to the nephrectomized animal establishes high plasma levels with relatively insignificant penetration into the vitreous. Under these circumstances, the secretion of trace doses out of the eye is unaltered despite the uphill concentration gradient.

Proving the existence of active transport of iodopyracet out of the rabbit eye does not necessarily localize the site of the process. The demonstration that the rabbit ciliary body accumulates iodopyracet in vitro against a concentration gradient with analogous saturation and inhibition characteristics[6] strongly suggests that this is one in vivo transport site. However, concurrent participation of other tissues such as retina and choroid is not ruled out.

Secretion of iodopyracet and related organic anions into the urine by the renal tubular epithelium has long been known.[6,7] The similarity between the renal and ocular mechanisms in terms of saturation and inhibition phenomena is readily apparent. A further mechanistic analogy may be drawn between the absence of iodopyracet from the anterior chamber at trace levels in the vitreous and its absence from the renal vein at low plasma levels.

Cell membranes are relatively impermeable to organic electrolytes actively transported by the kidney.[5] Comparable to this is the poor penetration of iodopyracet into the intraocular fluids. Systemic administration of probenecid did not alter the ocular barrier, demonstrating that it is not dependent upon the secretory function.

Intravitreal trace and saturation doses of iodopyracet were administered to rabbits with body temperatures reduced to 20°C. by immersion hypothermia.[8] Under these conditions there was a marked decline in both the turnover rate for trace doses and the maximum transport capacity. Because in vivo hypothermia introduces many variables such as alterations in blood flow and diffusion rates, the data cannot be interpreted as a temperature coefficient for the transport process.

Experiments analogous to the trace dose, saturation dose and systemic inhibition studies have been performed in the monkey and guinea pig. It is clear that there is an ocular transport mechanism in both of these species which secretes iodopyracet out of the eye and can be blocked by the systemic administration of probenecid. It is of interest that this process exists in the primate and is directed from intraocular fluid to blood, as in the rabbit. At the present time the possible physiologic role of this system remains a matter for speculation.

Summary

1. Intravitreal trace doses of iodopyracet were lost rapidly from the rabbit eye without appearance in the anterior chamber.

2. At higher concentrations of iodopyracet saturation phenomena were observed, resulting in a diminished rate of loss from the eye and accumulation in the anterior chamber.

3. Intravitreal administration of related organic anions led to similar reduction in rate of loss from the eye with accumulation in the anterior chamber.

4. Systemic probenecid resulted in similar inhibition.

5. The process was not altered by imposition of an uphill concentration gradient.

6. It was concluded that these data characterize an ocular transport mechanism which secretes iodopyracet out of the eye.

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ACKNOWLEDGMENT

We wish to acknowledge gratefully the technical assistance of Mrs. Tommie-Ray Tracy and Miss Carol Fritz.

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DISCUSSION

DR. V. EVERETT KINSEY (Detroit): It is rare that one has an opportunity of listening to two such original papers. The observation of a system that is capable of actively transporting certain anions out of the eye provides a basis for interesting speculation as to its physiologic significance.

Dr. Becker pointed out that these experiments were similar in some ways to those made by Friedenwald and Stiehler in 1938. The latter investigators showed that crystal violet would accumulate in the ciliary epithelium and that bromophenol blue would accumulate in the stroma of the ciliary process. Dr. Friedenwald thought that this phenomenon was associated with an electron transport system, which in turn he believed was concerned with the formation of aqueous humor.

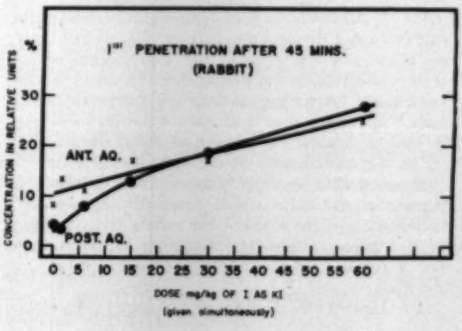
Dr. Becker sees no such implications in his studies. Just what significance his observations do have, however, is not known at present, but one possibility is that the excretory system might function in detoxication.

On the other hand, nature is frequently parsimonious, and it may be that although the ciliary epithelial cells function similarly to those in the kidney tubule, no useful function is performed in the eye.

In any event, these findings open broad new possibilities for experimentation. One of the possibilities—work that Dr. Becker has not had time to report—concerns the transport of iodide out of the eye. Dr. D.V.N. Reddy and I were stimulated on the basis of this work to determine whether iodine like Diodrast is excluded from the eye. If iodide is actively transported out of the eye, one might expect that iodide can enter the eye only with difficulty, just as is the case with Diodrast, para aminohippuric acid, and so forth.

May I show some data which supports this idea (fig. 1). Radioactive I^{131} was injected intravenously into rabbits along with doses of ordinary KI to saturate any transport system involved in keeping iodide out of the posterior and/or anterior chambers. The dose of KI varied from 0 to 55 mg./kg. of I, as KI. The concentration of the tracer (I^{131}) in the posterior and anterior chambers was determined after 45 minutes.

The figure shows that when no KI is given very



little I^{131} gets into the posterior aqueous. However, as the amount of KI increases the amount penetrating also increases rapidly. Appreciable amounts of I^{131} get into the aqueous of the anterior chamber even when no KI is given the animals. The effect of the KI is much less pronounced in the case of the anterior chamber, in fact, the increased concentration found after KI administration may only reflect increased contribution of I^{131} entering the anterior chamber from the posterior chamber.

The increased concentration in the posterior chamber with increasing quantities of KI we believe suggests that the excretory system becomes saturated, just as Dr. Becker observed in the case of the organic anions.

PAHA (0.5 gm./kg.) or Benemid (0.25 gm./kg.) were also administered with I^{131} (no KI). These compounds resulted in little if any increase in the amount of I^{131} entering the posterior chamber and had no effect on the rate of entrance in the anterior chamber.

This is but one example of the many possible new approaches to the study of ion transport brought out by the work of Dr. Becker and Dr. Forbes. Again I wish to commend them on the excellence of their papers.

DR. MAURICE E. LANGHAM (Baltimore): I would like to ask Dr. Becker two questions.

In listening to this stimulating communication my thoughts turned immediately to a possible correlation of his observations with the transfer into or out of the eye of two physiological anions, ascorbate and lactate. As we are well aware ascorbic acid is taken from the blood plasma by the cells of the ciliary processes and accumulated in the aqueous humour. In addition, it has been found that the concentration of lactate in the aqueous humour may itself be modified in an inverse manner by the concentration of ascorbic acid in the eye.

Secondly, I would appreciate Dr. Becker's comments on the specificity of this type of transport. The dye fluorescein which also exists principally as an organic anion at body pH may be injected in minute amounts into the vitreous humour and yet pass through the posterior chamber into the anterior chamber. Therefore, on this basis, it would appear that the uptake of p. amino hippuric acid is a very specific type of transport mechanism.

DR. MAURICE KADIN (Chicago): The iris in the rabbit cannot be separated from the ciliary body, so I am wondering about the contribution of the weight involved in the preparation. In weighing the iris-ciliary body preparations of many rabbits I have found that they will vary from 30 to 80 mg. in weight. I have also wondered about the possibility of the contribution of the vascular contents.

Some studies recently done with I^{131} labeled albumin in circulation with freeze-drying and determination of the ratio of the radioactivity per mg. of dry tissue, might be interesting in determining the vascular contribution to the concentrations in the eye.

DR. HERBERT E. KAUFMAN (Boston): To elaborate further the exciting concept of the eye as an organ of excretion, it may be possible to define with some degree of certainty exactly where the excretion of diodrast is occurring by means of autoradiography with radioactive diodrast. I am sure that similar studies must already be under way.

In addition, as Dr. Becker is aware, Dr. Pappenheimer has recently demonstrated the excretion of diodrast by the goat central nervous system. In perfusing fluid into the lateral ventricle of the goat and removing fluid from one of the basal cisterns, he found that diodrast is removed from the fluid by the central nervous system. This excretion appears to be accomplished by the arachnoid.

Since goat arachnoid is easly obtained, I wonder whether arachnoid might be used in a way similar to frog skin or toad bladder for in vitro studies.

DR. BERNARD BECKER (closing): I would like to thank the various discussers for their kind comments and suggestions.

With reference to Dr. Kinsey's slide about iodide, this is apart from the present paper, but we have been able to demonstrate a system which secretes iodide out of the rabbit eye. Interestingly enough, this system does not appear to be related to the diodrast transport system. It demonstrates, as Dr. Kinsey has indicated, how complex the secretion of aqueous humor really is.

The "iodide pump" (and this provides a very excellent correlation with Dr. Kinsey's experiments) can also be saturated by intravitreous or systemic administration of iodide.

In answer to Dr. Langham, we have found no relationship between these transport systems and such constitutents of the aqueous humor as ascorbate and lactate. Unfortunately ascorbate and lactate are not inhibitors of this system either in vivo or in vitro. Furthermore, the complete suppression of this system with massive doses of Benemid does not alter the ascorbate concentration of the rabbit aqueous nor does it alter its turnover. Therefore, we must say that in addition to our ordinary concepts about transport of organic anions, we have a diodrast transport system out of the eye, an iodide transport system out of the eye, and an ascorbate system into the eye, and these three do not at the moment seem to be related.

Fluorescein is not a competitive inhibitor of this system. This is quite interesting because, as you know, fluorescein is not excreted by the kidney very well but is put out by the liver. If one is permitted to make general statements, those substances that seem to be selectively secreted by the liver are not transported out by the eye; those that are secreted by the kidney are the ones that compete for the diodrast transport system of the eye. Again, I have no idea what this means.

In answer to Dr. Kadin, it is true (and I should have made the point), that we have evidence that the accumulation of labeled diodrast in vitro is largely in the ciliary body, and that we are using a ciliary body-iris preparation. As best I can judge, it means that the ratio of 10 to 15 times

media measured will have to be multiplied by another factor of 10 or 12 in order to estimate the true accumulation in the ciliary body. This would mean (just as has been demonstrated in the renal tubule) that the true accumulation in the ciliary body itself probably is some 100 to 150 times that of the media.

The contribution of blood in the ciliary body in vitro has been of concern to us. All I can say is that diodrast is not accumulated in the red cell as far as we are able to tell.

In answer to Dr. Kaufman, I learned at the recent Macy meeting about Dr Pappenheimer's work. I think the correlation is excellent. The suggestion of using the arachnoid is intriguing. As you know, substances like iodide, salicylates, and other organic anions, are excluded from the cerebrospinal fluid, and various elaborate hypotheses have been used to explain their exclusion. Dr. Pappenheimer has demonstrated that some of these are secretted out of the spinal fluid. The findings could lead to more teleologic and other speculative reasoning, but I will refrain from discussing such aspects at the moment.

NIGHT BLINDNESS, DARK ADAPTATION, AND THE ELECTRORETINOGRAM*

JOHN E. DOWLING
Cambridge, Massachusetts

INTRODUCTION

Recent studies on the origin of the electroretinogram indicate that these potentials arise in the visual and bipolar cell layers.[1, 2] The ERG response is easily elicited and provides a ready opportunity for studying visual cell function under both normal and pathologic conditions. In the past few years, we have been using the electroretinogram to evaluate visual responses in vitamin A deficiency[3, 4, 4a] and in light and dark adaptation. This paper will summarize the results of these experiments, discuss the electroretinographic findings and correlate these observations with biochemical and histologic data.

MATERIALS AND METHODS

Electroretinography. Albino rats from the Harvard colony were used in all these studies. The animals were lightly anesthetized with pentobarbital (10 to 15 mg.) and secured to a board fitted with a head holder. The eyes were exposed by drawing the eyelids apart with cotton sutures. The electroretinogram was recorded with cotton wick electrodes, soaked in Ringer solution and connected with a Grass amplifier and Dumont oscilloscope fitted with camera attachment. Both direct and capacity-coupled amplification were used, and the responses were similar in either case.** The eye was stimulated with .02 second flashes of white light from a tungsten filament lamp or zirconium arc. The intensity of the test flash was continuously controlled with circular photographic wedges, supplemented with neutral density filters.

In a typical experiment, the threshold intensity required to elicit an electroretinogram was first determined by flashing the test light every few seconds at increasing intensities until the response could be celarly distinguished from the baseline fluctuations on the oscilloscope trace. The electroretinogram was then recorded as a function of light intensity over a range of five to seven log units.

Rhodopsin Measurements. For measurement of rhodopsin, two retinas were hardened in alum for at least 15 minutes, washed twice with water and once with buffer, and were extracted overnight in 0.2 ml. of 2.0 percent digitonin. After centrifugation, 0.01 ml. of 1M NH_2OH was added to the clear extract and the absorption spectrum was measured. The solution was then bleached with white light for five minutes and the absorption spectrum remeasured. The differ-

* This investigation has been supported in part by grants from the National Science Foundation and U. S. Public Health Service.

** The slow c-wave of the ERG, somewhat compromised by the use of capacity coupled amplification, is seen infrequently in the rat ERG and consequently was not looked for.

ence in extinction at 500 mμ. is a measure of the concentration of rhodopsin.

Vitamin A Deficiency. For the vitamin-A deficiency studies, weanling rats were raised on the standard U.S.P. Vitamin A test diet. The controls were either animals raised on the vitamin A-free diet supplemented with vitamin A, or animals raised on the complete laboratory ration. The supplements of vitamin A and vitamin A acid were dissolved in vegetable oil and fed orally three times a week at a level of 50 μg. per day.

Light and Dark Adaptation. Groups of normal rats were dark-adapted overnight, and then light-adapted for one-half hour in a white porcelain pan brightly illuminated with three 100-watt flood lamps. After the lights were extinguished, individual animals were selected at various times, anesthetized lightly with pentobarbital and their electroretinograms recorded. The anesthetic was given five to 10 minutes before the time of measurement. For the initial measurement, it was therefore administered during the last few minutes of light adaptation. After induction of anesthesia, an animal could be readied and an electroretinographic threshold determined within three minutes. The rhodopsin measurements were made the following day on the same or different groups of animals. For this, the animals were light-adapted and anesthetized exactly as before. At the appropriate times during dark adaptation, the eyes were enucleated and analyzed for rhodopsin.

Results

Vitamin-A Deficiency. When weanling rats are placed on a vitamin A-free diet, they continue to gain weight, appear healthy, and exhibit normal visual responses for three to four weeks. During this time, no changes are detectable in these animals except for the steady depletion of liver stores of the vitamin. In about the fifth week, the liver stores are exhausted, and with this the blood vitamin A falls precipitously. At the same time the rhodopsin concentration in the retina begins to fall and this marks the beginning of night blindness. Within a few days more, the animals also cease to gain weight.

The animals continue to lose rhodopsin throughout the experiment. In about the seventh week they begin to lose weight rapidly and develop severe signs of the deficiency. By the end of the eighth or ninth week, most of the animals have died.

Figure 1 shows the electroretinograms recorded from pairs of dark-adapted animals during the course of such an experiment. At the top of the figure is shown the week in which the responses were recorded. The second line indicates the average rhodopsin content of these eyes, expressed as percent of the normal. On the third line is shown the logarithm of the electroretinographic threshold, the lowest luminance of light that evoked a perceptible electroretinogram. The normal threshold is set arbitrarily as one, so that the normal log threshold equals zero. The ERG's are recorded over a range of test luminances of five log units (100,000 times).

The normal electroretinogram of the rat is typical of those obtained from rod-dominated mammalian eyes. At low light intensity, one sees only cornea-positive b-waves, which gradually increase in potential with increase of intensity of stimulation. At higher intensities, the cornea-negative a-wave becomes increasingly prominent until it approaches the size of the b-wave. At the highest intensities, the electroretinogram measured from the trough of the a-wave to the peak of the b-wave is equal to about 1,500 μv.

During depletion of the liver stores of vitamin A, no changes are noted in the electroretinogram. The first two rows of responses, therefore, compare the electroretinograms of a control animal (week 0) and an animal which had been on the deficient diet for four weeks.

In the fifth week of the experiment, the rhodopsin level in the retina declines and with this the electroretinogram shows marked changes. The threshold is raised about 1.15 log unit or about 14 times. As the deficiency progresses, the concentration of rhodopsin continues to fall and the threshold to rise, so that in the eighth week, when the rhodopsin

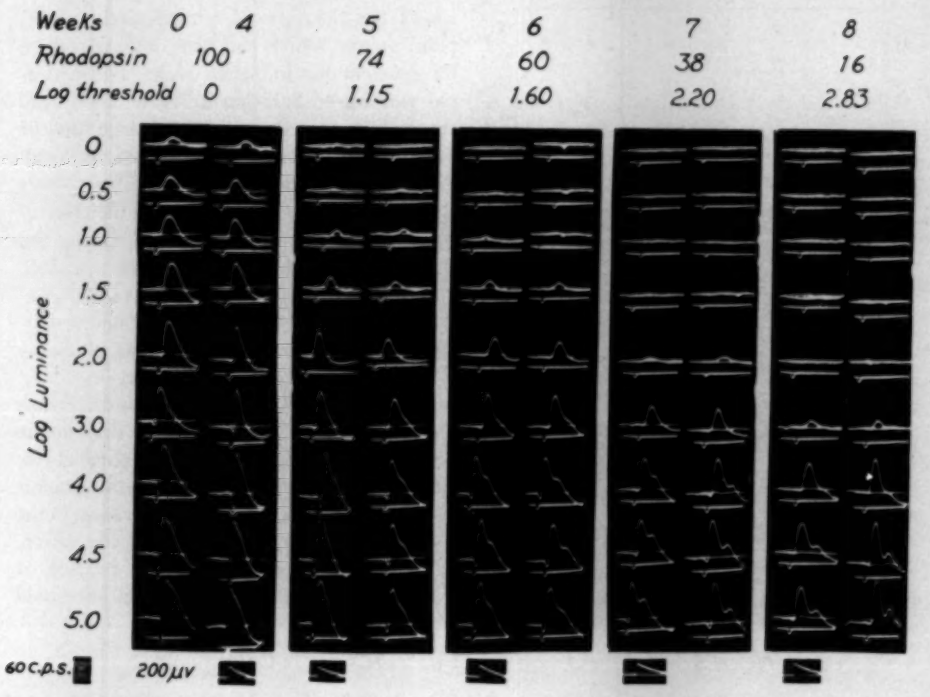


Fig. 1 (Dowling). Effects of vitamin-A deficiency on the electroretinogram. The top three lines show the number of weeks on the deficient diet; the rhodopsin content of the retina expressed as percent of normal; and the logarithm of the threshold, the lowest luminance needed to evoke a perceptible electroretinogram. The average threshold is set arbitrarily at one; therefore log threshold = 0. The electroretinograms are evoked with .02 sec. flashes of white light over a range of extending five log units. The first two rows of electroretinograms show responses from a control animal (week 0) and those from an animal on the diet for four weeks, when the responses are still normal. Thereafter, records are shown from a pair of animals each week. As the rhodopsin declines, the electroretinographic threshold rises and the electroretinogram changes characteristically in form.

is reduced to only 16 percent of normal, the threshold is raised 2.8 log units, or about 680 times.

In the course of the deficiency, the form of the ERG changes also. The a-wave, which in the normal rat is especially conspicuous at the higher intensities, is more severely depressed than the b-wave and gradually disappears. By the end of the eighth week, no a-wave is seen, although a b-wave of considerable size is still recorded at high light intensities. Furthermore, as the deficiency progresses, the b-wave separates into two positive peaks. This secondary wave, seen in normal animals as a slight inflection at the top of the b-wave, is delayed longer and longer as the deficiency progresses, and by the seventh week has separated off as a second wave. The appearance of this second wave is characteristic of vitamin A deficiency and has not been seen in the rat electroretinogram under other circumstances.

Figure 2 compares the rise of electroretinographic threshold and decrease in rhodopsin content with time on the diet. No change is seen in either rhodopsin content or log threshold until the liver stores are depleted (three to four weeks); then beginning in the fifth week, the log threshold rises in parallel with the decrease of rhodopsin concentration. The parallelism between fall of rhodopsin concentration and rise of electroretinographic

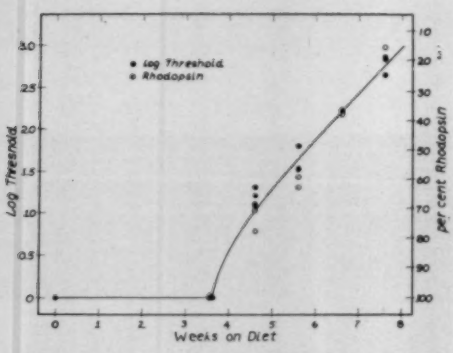


Fig. 2 (Dowling). Development of night blindness and decline of rhodopsin concentration with time on the vitamin A-deficient diet. The logarithm of the electroretinographic threshold rises in parallel with the decline of rhodopsin concentration.

threshold continues through the course of the deficiency.

When vitamin A is given to a deficient animal, it recovers unless the animal has deteriorated too greatly physically. Figure 3 shows the recovery of a moderately night-blind animal which had been maintained on the deficient diet for seven weeks. The threshold was raised 2.05 log units, or about 100 times. This animal was given a large dose of vitamin A by intraperitoneal injection (1 mg. vitamin A dissolved in vegetable oil). Over the next few days, the changes in the electroretinogram reversed those noted during the development of the deficiency. The threshold returned to normal in about 60 hours, the a-wave increased in size to its former prominence, and the secondary peak was gradually assimiliated back into the b-wave.

Light and Dark Adaptation. We have seen that in vitamin-A deficiency, the decrease in rhodopsin is accompanied by the parallel rise of the logarithm of the electroretinographic threshold. The rhodopsin concentration in the eye can also be lowered with light adaptation. It is of interest to compare the relation of rhodopsin concentration and visual threshold

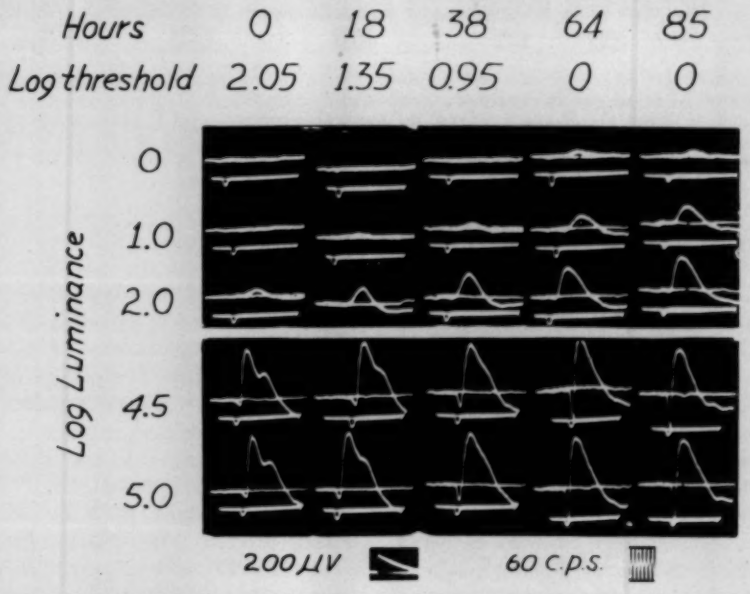


Fig. 3 (Dowling). Recovery from night blindness on administration of vitamin A. Following intraperitoneal injection of a large dose of vitamin A, the electroretinographic threshold returns within 64 hours to normal (log threshold 0). The electroretinogram reverses all the changes which have accompanied the development of night blindness.

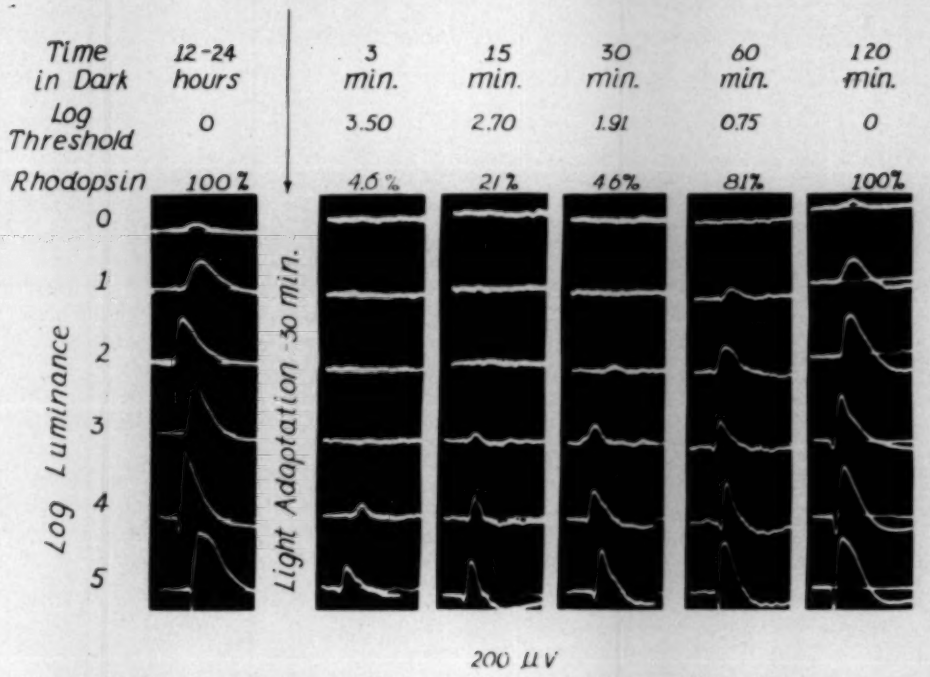


Fig. 4 (Dowling). The electroretinographic responses during dark adaptation in the normal rat. The top three lines show the time in the dark: the logarithm of the electroretinographic threshold, the lowest luminance necessary to evoke a perceptible electroretinogram, and the rhodopsin content expressed as percentage of dark-adapted level. The electroretinograms are evoked with .02 second flashes of white light over a luminance range of five log units. The first row of electroretinograms shows the responses of a control animal, dark adapted overnight. After light adaptation, the electroretinographic threshold is raised about 3.5 log units, and the responses at high luminances are diminished in size. With time in the dark, the threshold returns to normal, and the responses at higher luminances assume the dark-adapted form.

during dark adaptation with that found in vitamin-A deficiency.

For this, groups of rats were intensely light adapted for one-half hour, and then placed in the dark. At selected times during dark adaptation, an animal was anesthetized and its ERG recorded. Subsequently, parallel experiments were performed which determined rhodopsin levels during the dark adaptation period (for experimental details, see Methods).

Figure 4 shows the electroretinograms recorded in such an experiment over a luminance range of five log units. At the top of the figure is noted the time in the dark after light adaptation. On the next line is indicated the logarithm of the electroretinographic threshold, the lowest luminance of light which evoked a perceptible electroretinogram. The normal threshold is again set arbitrarily at one, so that log threshold is zero. The third line shows the rhodopsin content at that level of dark adaptation, expressed as percentage of the completely dark-adapted level.

The first row of electroretinograms shows the responses of a control animal which had been dark-adapted overnight. After 30 minutes of light adaptation, the threshold, recorded after three minutes in the dark, was raised about 3.50 log units (about 3,000 times). The rhodopsin concentration at this

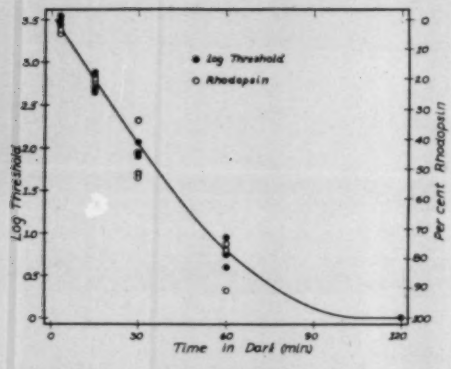


Fig. 5 (Dowling). Dark adaptation in the normal rat. Following light adaptation, the fall of the logarithm of the electroretinographic threshold parallels the rise in concentration of rhodopsin. Both the threshold and rhodopsin concentration are back to the dark-adapted levels in about 100 minutes.

point was reduced to four to five percent of the dark-adapted level. With time in the dark, the sensitivity of the eye returned gradually to the dark-adapted state, in parallel with the rhodopsin concentration. Dark adaptation was completed in about 100 minutes.

The changes in form of the electroretinogram characteristic of vitamin A deficiency are not observed when the rhodopsin content is lowered to the same extent as the result of light adaptation. In the early stages of dark adaptation, both a- and b-waves are depressed and the b-wave remains a single peak.

When the electroretinographic thresholds and rhodopsin concentration are compared during dark adaptation (fig. 5), it is seen that the log threshold falls in parallel with the rise in rhodopsin concentration. It will be remembered that a similar relationship holds during vitamin-A deficiency, where the decrease in rhodopsin is paralleled by a rise in threshold. Figure 6 shows the relationships during dark adaptation and vitamin-A deficiency to be identical. It is clear that the logarithm of the visual threshold even under these different conditions, rises linearly as the concentration of visual pigment falls. This relationship is expressed by the equation, Log $(I_t/I_o = 3.6(R_o - R_t)/R_o$, in which I_o and R_o are respectively the threshold and rhodopsin concentration in dark-adapted control animals, and I_t and R_t are respectively the threshold and rhodopsin concentrations in vitamin-A deficient or partly dark-adapted animals.

Vitamin-A Acid. Throughout most of the course of vitamin-A deficiency, the histology of the retina appears normal. In the terminal stages of the deficiency (in about the eighth week), when the animals are losing weight rapidly and show severe signs of the deficiency, the retina begins to degenerate.[3, 5, 6] This deterioration is not confined to specific structures but involves the whole retina. Indeed, at this time tissues throughout the animal are deteriorating.

Vitamin-A acid, when fed to rats on a deficient diet, maintains them in good health and prevents general tissue deterioration. The acid, however, is not reduced in vivo to either retinene (vitamin-A aldehyde) or vitamin A, which are necessary for vision; the animals therefore become highly night blind, and eventually completely blind.[4]

We have maintained animals for 10 months on a vitamin-A deficient diet, supplemented with vitamin A acid. Such animals never show any general somatic symptoms of the deficiency.

Figure 7 shows measurements of the elec-

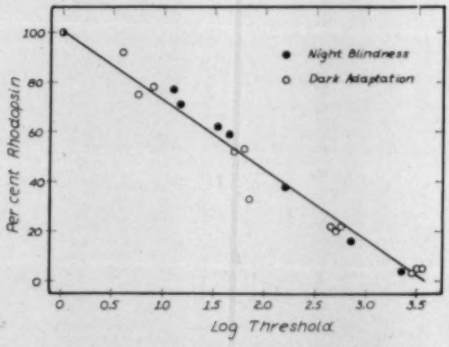


Fig. 6 (Dowling). The relation between rhodopsin content of the retina and the visual threshold, in animals night blind due to vitamin A deficiency, and in normal animals dark adapting after exposure to bright light. In both instances, the same relationship is observed: the log threshold rises linearly with fall in rhodopsin concentration.

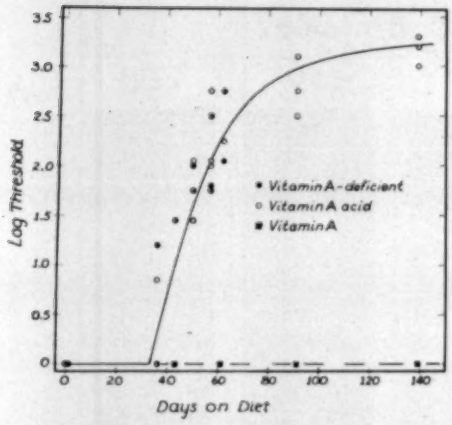


Fig. 7 (Dowling). Electroretinographic thresholds of animals kept on a vitamin A-deficient diet and supplemented with vitamin A dissolved in vegetable oil, vitamin A acid in vegetable oil, and vegetable oil alone (vitamin A-deficient). The threshold is the smallest luminance need to evoke a perceptible electroretinogram. The normal threshold is arbitrarily given the value 1.0 (log threshold = 0). The animals supplemented with vitamin A have normal thresholds throughout the experiment. In the animals supplemented with vitamin A acid, the threshold rises as soon and as rapidly as those receiving just vegetable oil. By the end of the ninth week, all the latter group have died. The vitamin A acid animals remain well, and become more night-blind. The thresholds gradually level off after 100-120 days at between 3.25 and 3.50 log units above normal.

troretinographic threshold from three groups of animals, raised simultaneously on the vitamin-A deficient diet. One group was supplemented with vitamin A acid dissolved in vegetable oil; the second with vitamin A, also in vegetable oil; and the third with vegetable oil alone (for details, see Methods). The animals fed vitamin A show normal electroretinographic thresholds and responses throughout the experiment. Those fed vitamin A acid or vegetable oil have normal thresholds for the first four weeks of the experiment, corresponding to the depletion of vitamin A stores from the body. Beginning in the fifth week, both groups develop night blindness, and at about the same rate (also at about the same rate as the animals shown in fig. 2).

After about eight weeks, the animals receiving vegetable oil alone have died, whereas those receiving vitamin A acid continue to grow but become increasingly night-blind. The electroretinographic thresholds of these animals, however, do not rise indefinitely, but level off gradually after 100 to 120 days at about 3.25 to 3.50 log units above the normal threshold. Their retinas contain about 1 to 3 percent of the normal rhodopsin concentration.

Figure 8 shows the electroretinograms of animals maintained on vitamin A acid, recorded over a range of luminances of seven log units. After 28 days on the diet, the animals have normal ERG responses. After 56 days, they give electroretinographic responses typical of severe vitamin-A deficiency (fig. 1). The threshold is raised about three log units; the a-wave is reduced to a just perceptible level even at the highest luminances; and the b-wave has separated into two positive peaks.

After 139 days on the diet, the threshold is raised only slightly further, to about 3.5 log units. However, with superthreshold stimuli, the electroretinographic response is considerably diminished in size. Even at the highest luminances the electroretinogram consists of only a small, slow b-wave with no fine structure or a-wave. At this stage the retina appears to be losing its ability to generate an ERG, though with little further rise in threshold (fig. 7). As the diet is continued, the electroretinograms of these animals become smaller and smaller, and by 288 days no electrical responses can be recorded at all. At this stage, the animals appear completely blind.

Figure 9 shows the retinal histology associated with the ERG responses shown in Figure 8. The control is an animal maintained for 10 months on the vitamin-A free diet, supplemented with vitamin A. The structure of its retina is perfectly normal. In the animals maintained on vitamin-A acid, the first histologic change is seen after about two months (fig. 9b), at about the same time that histologic changes are seen in unsupplemented vitamin A-deficient animals. However, where in vitamin-A deficiency all retinal tissues may degenerate, here the changes

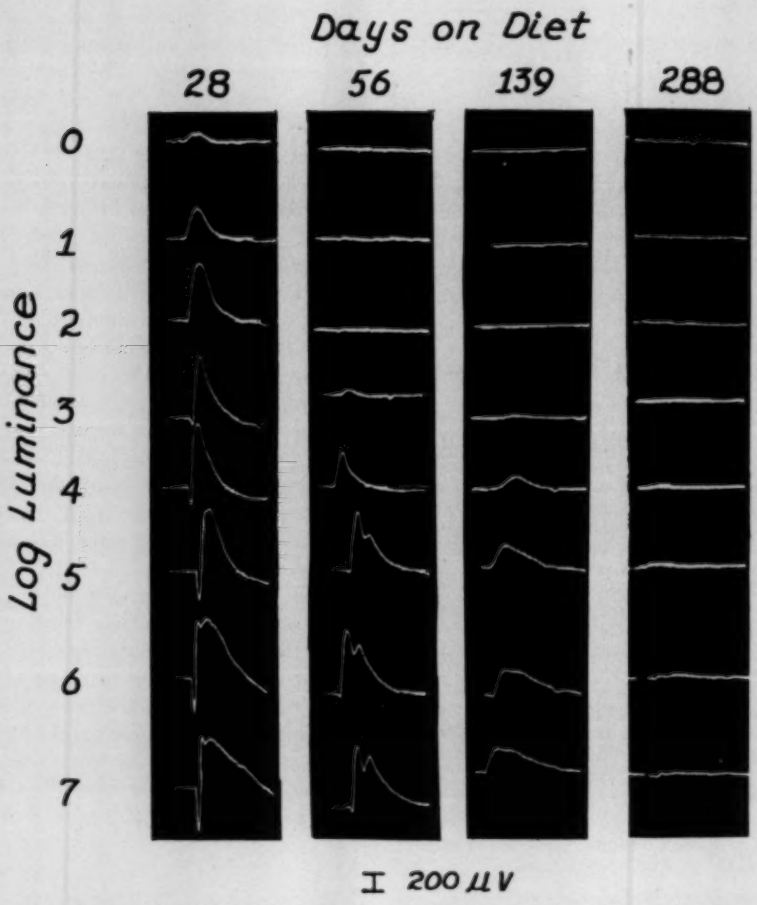


Fig. 8 (Dowling). Electroretinograms of animals supplemented with vitamin A acid. The electroretinograms are evoked with .02 sec. flashes of white light at luminances ranging over seven log units. After 28 days, the responses are still normal. After 56 days, the electroretinogram displays the characteristic changes of vitamin A deficiency: the threshold has risen about 2.5 log units, the a-wave is reduced to just perceptible level, and the b-wave has separated into two positive peaks. By the 139th day, the threshold has risen slightly further, but now at the higher luminances the electroretinogram is reduced in size. The retina is losing its ability to generate an electroretinogram. With time, the responses become smaller and smaller, and by the 288th day, no responses can be elicited at the highest luminances. The animal is now blind.

are limited specifically to the visual cells. Initially, the rod outer segments stain less intensely than normal, and appear broken and disorganized, whereas the rest of the visual cell appears normal, along with the rest of the retina and pigment epithelium.

After six months on the diet (fig. 9c), the outer segments have almost completely disappeared; only scattered fragments remain. Now also the inner segments and visual cell nuclei are reduced to about half their former number. The rest of the retina—bipolar cells, ganglion cells and pigment epithelium—appears normal. After 10 months (fig. 9d), the visual cells are almost entirely gone. No inner or outer segments are visible, and the

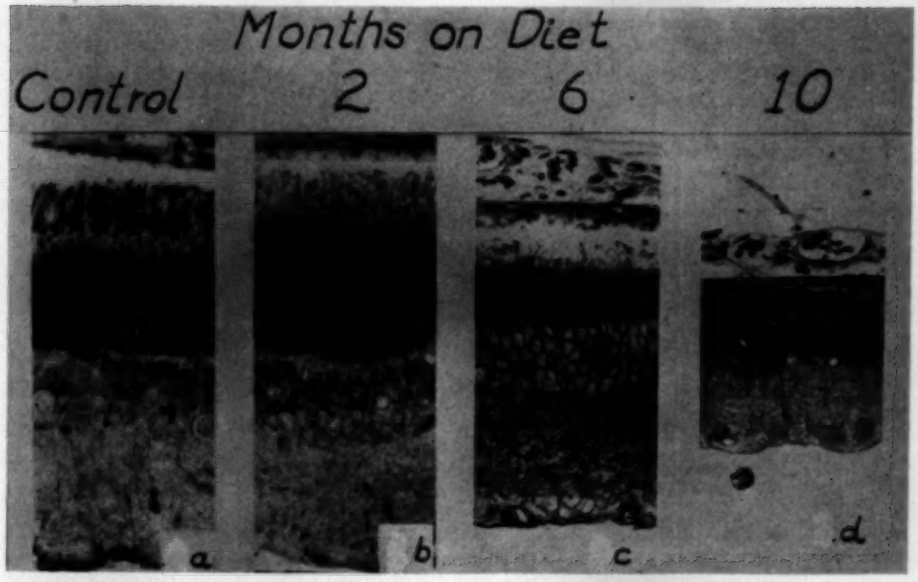


Fig. 9 (Dowling). Retinal histology of rats raised on vitamin A-free diets and supplemented with vitamin A acid.

a The retina from a control animal that had been raised for 10 months on a vitamin A-free diet supplemented with vitamin A. The structure is entirely normal.

b The retina of an animal raised for 2 months on vitamin A-free diet supplemented with vitamin A acid. The primary change has occurred in the outer segments which are disoriented and stain less intensely. The rest of the visual cell appears normal, as do the other layers of the retina.

c After six months, the outer segments have almost entirely disappeared. The inner segments and visual cell nuclei are reduced to about half the normal number. The rest of the retina and the pigment epithelium appear normal.

d Retina from an animal maintained for 10 months on the diet supplemented with vitamin A acid. The visual cells have disappeared, except for one irregular row of visual cell nuclei. Other parts of the retina appear normal.

layer of visual cell nuclei is reduced to one incomplete row. Again the rest of the retina and the pigment epithelium appear normal.

When vitamin A is given to the animals on a vitamin-A deficient diet supplemented with vitamin-A acid, visual recovery occurs if the retinal degeneration has not progressed too far. After six months on this regime, when half the visual cell nuclei and inner segments are still present, recovery occurs in those cells that remain. By 10 months, when only one incomplete layer of visual cell nuclei remains, no recovery is observed even after weeks of feeding vitamin A.

Figure 10 shows the recovery of the electroretinogram and histology of an animal which had been maintained on vitamin A acid for six and one-half months. For this experiment, three littermates were raised on a vitamin A-free diet. The control was fed vitamin A, and the other two animals, vitamin A acid. Sixteen days before the end of the experiment, one of the vitamin-A acid animals was fed a large dose of vitamin A (1 mg.) and then given further supplements of vitamin A throughout the experiment.

The retinal structure of the control animal is entirely normal (fig. 10a). The retina of the animal maintained on vitamin A acid for six and one-half months is typical of such animals: most of the outer segments are gone, and the inner segments and visual cell nuclei are reduced to about half their normal number (fig. 10b). The animal fed vitamin A has regenerated new outer segments, normal in appearance. However, there has been no

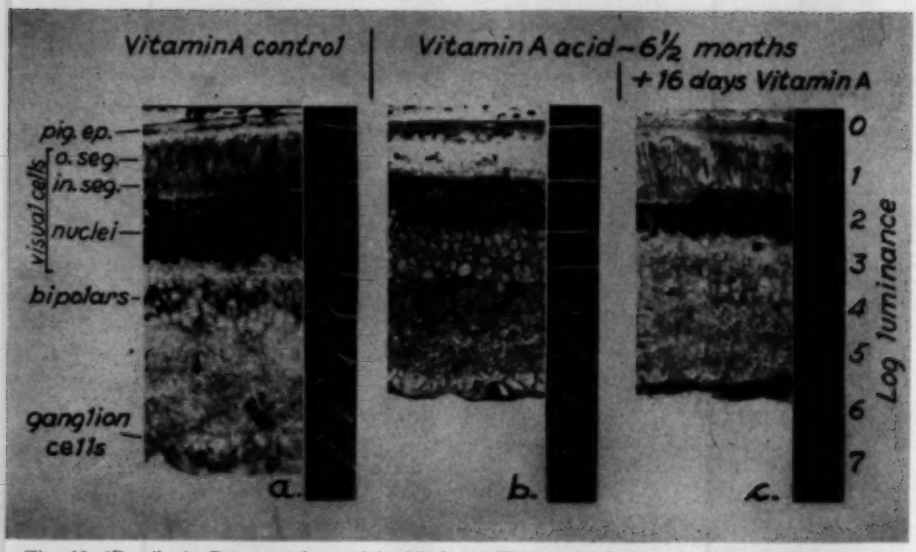


Fig. 10 (Dowling). Recovery from night blindness. Three animals were kept for 6½ months on a vitamin A-deficient diet supplemented with vitamin A (control) and vitamin A acid. The retinal histology and electroretinograms of the control (a) are normal. 16 days prior to the end of the experiment one to the other animals was fed a large dose of vitamin A. His electroretinograms before being fed vitamin A are shown (b) along with a retinal section from the third animal, similarly night blind. The electroretinograms show a high degree of night blindness. The retina shows an almost complete loss of outer segments and reduction of nuclei to about half the normal number. After recovery (c) the remaining cells have regenerated new outer segments, but there has been no increase of visual cell number. The electroretinograms closely resemble the normal in threshold level and form, but remain much reduced in size. The reduction in size is permanent and probably due to the loss of visual cells.

increase in visual cell number, so that there are only about half the normal number of outer segments (fig. 10c).

The ERG's of these animals, recorded over seven log units of luminance, are included in the figure. The electroretinogram of the control is normal. The electroretinogram of the vitamin-A acid animal before being fed vitamin A, shows the changes typical of long-term deficiency. The threshold is raised more than three log units, there is no a-wave, and the b-wave has two peaks. The electroretinogram is also considerably diminished in size. After recovery, the electroretinographic threshold of this animal has returned almost to normal. The residual, permanent rise in threshold (about 0.25 log unit) is undoubtedly due to the loss in the number of visual cells. This may be analogous to the rise in threshold observed upon reduction in the area of the visual field. The electroretinogram has also regained its normal shape. However, with superthreshold stimuli, the size of the electroretinogram remains diminished to about half that of the control electroretinogram.

Discussion

We have found a linear relation to hold between the logarithm of the electroretinographic threshold and the concentration of rhodopsin during dark adaptation and in night blindness caused by vitamin-A deficiency. Recently Rushton[7] reported a similar relationship between the psychophysical threshold and rhodopsin concentration in the living human eye during dark adaptation.

Previous attempts to correlate visual pigment concentrations with electroretinographic responses during dark adaptation have not been entirely successful.[5,9,10] All of these experiments, however, have attempted to correlate the visual pigment concentration with

the increase in size of the b-wave potential elicited with a constant test flash. As can be seen from Figure 4, no simple proportionality exists between the height of the b-wave and the visual pigment concentration. If the test light is too dim, for example, such as log luminance 1 or 2 in Figure 4, one will not even begin to record an electroretinogram until considerable quantities of rhodopsin have accumulated in the retina. Perhaps this is why Granit, Munsterhjelm, and Zewi failed to record an electroretinogram in the cat until 40 percent of the visual pigment had regenerated.[8] On the other hand, if the test stimulus is too bright, it seems likely that one would find that large fluctuations in rhodopsin had little effect on the size of the electroretinographic potential. It is preferable, therefore, to evaluate the sensitivity of the eye by measuring the light intensities required to evoke a constant ERG[11] or, as we have done, the threshold ERG.

A further difficulty with earlier measurements may involve the participation of cones during the early stages of dark adaptation. Since there is much less visual pigment in the cones than in the rods, extraction of visual pigment from rod-dominated retinas yields essentially only the rod pigment (rhodopsin). The electroretinogram, however, reflects rod and cone activity. Granit, Munsterhjelm, and Zewi (fig. 1 of ref. 8), comparing rhodopsin concentration and the size of b-wave elecited with a bright stimulus in the dark-adapting frog eye, found that for the first hour of the experiment, when the visual purple was regenerating from negligible values to 40 percent or so, the b-wave was of constant and considerable size. One wonders if these workers were not initially observing the responses of cones. In the rat, dark adaptation measured under our conditions is uncomplicated by cone responses.

We have seen that the rhodopsin concentration falls both in night blindness and upon light adaptation. The decline of rhodopsin in both cases is accompanied by a parallel rise of electroretinographic threshold. However, the electroretinograms elicited with super-threshold stimuli exhibit distinct differences in form in the two instances. After light adaptation, both the a- and b-waves are considerably depressed at all luminances. During the early stages of night blindness owing to vitamin A deficiency, however, the a-wave is depressed selectively compared with the b-wave. So for example, in the eighth week (fig. 2) or on the 56th day (fig. 7), the b-wave remains almost normal in size at high luminances, whereas the a-wave is reduced to a just perceptible level. At this time the retinal histology of the animals maintained on vitamin-A acid shows that the outer segments are deteriorating. This suggests that in night blindness owing to vitamin-A deficiency, the damage of outer segments may be related to the selective depression of the a-wave. (It has earlier been suggested for other reasons that the outer segments may be closely related to the a-wave.[1, 2]) The production of a b-wave rests ultimately upon excitation through the outer segments; yet this evidence suggests that a residual outer segment activity, associated with a greatly depressed a-wave, can still give rise, presumably at a different (inner) locus, to an almost undiminished b-wave.

The separation of the b-wave into two peaks is characteristic of the early stages of vitamin-A deficiency, and is not seen in the rat electroretinogram at any other time. In man, one sometimes finds double-peaked b-waves, especially after light adaptation.[12] The two peaks are ascribed to photopic and scotopic contributions to the electroretinogram. Histologic examination shows that rats possess some cones but their visual responses are hard to demonstrate. During dark adaptation, for example, one does not find a cone break or a double b-wave, and Graham and Riggs[13] were unable to find any evidence for the Purkinje phenomenon in the rat. Attempts to see if the two components present in the b-wave during vitamin-A deficiency might be due to a separation of photopic and scotopic responses have been altogether unsuccessful. The significance of the double b-wave therefore remains obscure.

As shown in Figure 8, the electroretinogram gradually disappears in animals maintained on vitamin-A acid. This disappearance parallels the degeneration of the inner portions of the visual cell (fig. 9). Our recovery experiments also suggest a close relation between the size of electroretinographic potential and visual cell number (fig. 10). After regeneration of new outer segments, the threshold of the electroretinogram is close to the normal. The electroretinogram has also assumed a more normal form. However, the size of the electroretinogram remains about half that of the normal, corresponding to the halving in the number of visual cells.

As shown in Figure 9, the degeneration of the visual cell in animals maintained on vitamin-A acid begins in the outer segments but eventually includes the whole cell. It is clear that loss of vitamin A and retinene in itself cannot account for these anatomic changes. However, one must remember that rhodopsin consists of retinene joined onto a protein, opsin.

In the past few years, it has become increasingly evident that opsin is stabilized by combination with its prosthetic group, retinene.[14,15,16] Vitamin-A deficiency permanently deprives opsin of its prosthetic group and may thereby render it unstable. The outer segments of the rods are composed in great part of opsin and degeneration of the opsin might cause the anatomical deterioration of the outer segments.

It is, however, less clear why the rest of the cell should deteriorate. The histology of the visual cell under these conditions has been studied both with the light and the electron microscope,[17] and while the outer segments show drastic internal changes, no histologic changes have been seen in the inner segments, visual cell nuclei, or synapses before they disappear. Furthermore, these structures do not disappear until the outer segments are almost gone. This suggests that perhaps the cessation of stimulation to the inner layers of the visual cells might cause their loss. Metabolic and structural integrity depend upon excitation, and examples of nerve cells which die upon loss of stimulation are known.[18] Also neurons will sometimes die when just their distal axon is cut,[18] and perhaps this is an analogous case: the visual cell may degenerate because its outer segment is lost. As an alternative, one might postulate that vitamin A plays a further, yet unknown, role in the visual cell, that vitamin-A acid cannot replace.

Eventually the retinas of the animals maintained on vitamin A acid come to resemble closely the pathology described for several hereditary conditions resulting in night blindness in man and animals. The defect in C3H mice [19,20] and retinitis pigmentosa[21] in man are two well-known instances. In both cases, the retina may appear almost identical with that shown in Figure 9d.

The similarity between the histologic changes in vitamin-A deficiency and retinitis pigmentosa have led certain workers to suggest that a local vitamin-A deficiency in the retina might be responsible for this disease.[21,22,23] However, a recent study on the electroretinographic responses of patients with retinitis pigmentosa[24] showed that in several cases the b-wave was lost very early in the disease, while the a-wave persisted after the b-wave had entirely disappeared. These changes are the opposite of those seen in vitamin-A deficient rats, in which the a-wave disappears before the b-wave. We have suggested that the a-wave reflects the activity of the outer segments of the visual cells, and if this is correct, it suggests that the defect in retinitis pigmentosa, may not be located in the outer segment and hence probably not directly involved with the visual cycle.

Summary

When weanling rats are placed on a vitamin-A deficient diet, the liver stores are first depleted of the vitamin. Then the blood vitamin A falls precipitously, and with this the rhodopsin content of the eye declines. The decline of rhodopsin is accompanied by the parallel rise of the logarithm of the electroretinographic threshold.

Light adaptation also decreases the rho-

dopsin content of the eye and similarly raises the electroretinographic threshold. During dark adaptation, the rhodopsin content of the eye increases in parallel with the fall of the logarithm of the electroretinographic threshold.

Vitamin-A acid prevents general tissue deterioration in animals raised on a vitamin-A deficient diet. The acid, however, is not reduced in vivo to either retinene (vitamin A aldehyde) or vitamin A, which are necessary for vision. Consequently these animals become extremely night blind and eventually completely blind.

The visual cells of these animals degenerate, and the electroretinographic responses disappear. Initially the outer segments degenerate, and this is accompanied by the loss of the a-wave of the electroretinogram. Somewhat later the rest of the visual cell degenerates, accompanied by the disappearance of the rest of the electroretinogram.

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Discussion

Dr. Werner K. Noell (Buffalo, New York): What we have just heard undoubtedly represents a major and most significant advance in retinal physiology and pathology, and it gives me a great deal of pleasure to congratulate the author on his excellent work.

Never in the past have all the relationships between vitamin A availability in retinal function been so extensively demonstrated and so beautifully demonstrated as we have seen and heard it today; and never before, I think, has anybody observed experimental retinal degeneration to proceed as slowly as in these experiments in which the systemic effect of vitamin A deficiency was prevented by feeding vitamin A acid.

For the first time, moreover, conclusive evidence has been obtained that the outer segments are capable of regeneration as long as structural damage does not proceed beyond the basal portion of the outer segment. The fact that the outer segments are capable of regeneration, perhaps in a similar way as the axone of the ganglion cell, should be of immense importance for ophthalmology, clinically and research-wise.

I am thinking not only of the repair of outer segment changes in detachment or in intoxication or deficiency, but I am also thinking of the initial stages of hereditary visual cell degeneration which we might be able to repair and recover if we knew where the metabolic lesion is.

[Slide] The similarity between hereditary visual cell degeneration as we observe it in mice and those which Dr. Dowling showed in vitamin A deficiency is very striking. The last part of the slide to the right shows the retina at the end of the degenerative process. Here is one row of visual cell nuclei remaining.

[Slide] It is also a pleasure to note that in all of this work concerning the electroretinogram Dr. Dowling's observations and ours with poison in retinal degeneration agree, namely, that the damage to the outer segment is associated with a drop in the C-3-H mouse in comparison to DBA, the normal mouse.

I think, however, it would cloud the issue if we would think that the A wave is generated by the outer segments themselves. I think matters are better stated in saying that the A wave depends more intimately on the function of the outer segment than other waves of the ERG, as, for instance, the b-wave.

[Slide] Another minor point concerns these double b-waves, which may appear to be characteristic of vitamin A deficiency but which we see very often when normal eyes are examined under nembutal. This is a double b-wave, a single flash of stimulus at the start of the cathode sweep, and you see this double b-wave and multiple b-waves here are very dependent upon the intensity of the stimulus. I think they are dependent upon an effect of nembutal upon the visual cell.

In closing, let me express again my congratulations to Dr. Dowling and my sincere admiration for his work.

Dr. W. A. H. Rushton (Cambridge, England): We all know there is a relation between the recovery of sight in dark adaptation and regeneration of pigment. We have known that for a long time; but we have all "forgotten" what the evidence is upon which this knowledge is based, and that is because there has never been any.

The striking thing about Dr. Dowling's communication is that it gives us such evidence. We see here not only that threshold is dependent upon rhodopsin concentration but that it is dependent in the simple way that Wald has suggested and, indeed, that Hecht suggested in 1920.

Most of us here are interested in human eyes and in human vision. It would therefore increase the value of this very fine piece of work if it were known that it applies also to the human eye. This follows from experiments that Fuortes, Gunkel and I have done in Dr. von Sallmann's laboratory during the last six months.

By the technique of retinal densitometry it is possible to measure physically the amount of rhodopsin in the human eye and to follow its regeneration after exposure to bright lights.

We have done this with normal subjects and with a rod monochromat and have found that the curves of rhodopsin regeneration were the same in both, namely an exponential with five minutes' time for half return.

The return of sensitivity of the monochromat—the conventional dark adaptation curve—had no cone branch which in normals hides the early part of the rod recovery. So we were able to plot the rod curve over a range of 6.5 log units. The curve so plotted was also an exponential with half-decay time of five minutes.

Thus in human rods the log threshold is proportional to the fraction of rhodopsin unregenerated, as Dowling has found in the rat, though the constants of proportion are different in the two species.

Dr. Mathew Alpern (Ann Arbor, Michigan): I have two questions I would like to ask Dr. Dowling. First, what kind of a mechanism does he propose to explain this linear relationship between the log of the threshold and the concentration of rhodopsin?

Second, how is he certain that this is a specific effect on the a-wave? Is it possible that the same effect could be produced merely by a shift of the whole curve along the intensity axis?

Since the a-wave comes in only some 2 or 3 log units above the b-wave threshold, might he not merely be showing the very small a-wave that finally comes in 2 or 3 log units above the very elevated b-wave threshold?

Dr. John E. Dowling (closing): I should like to thank Dr. Noell and Dr. Rushton for their very valuable discussion, and first comment on Dr. Noell's suggestion that the separation of the b-wave into two distinct peaks during vitamin A deficiency

may be due to the nembutal anaesthesia. We use nembutal in similar amounts in all of our ERG experiments, but only in vitamin A deficiency do we see double-peaked b-waves. The emergence and prominence of this second wave parallels the degree of deficiency not the amount of nembutal given, and unless the vitamin A deficient retina is peculiarly sensitive to nembutal, it seems doubtful to me that the anaesthesia could be causing this phenomenon.

In answer to Dr. Alpern, it seems to us that the a-wave is selectively depressed during vitamin A deficiency. Whereas we can record b-waves of considerable size in vitamin A deficiency, we no longer can evoke very prominent a-waves regardless of the illumination. In recent experiments using very strong lights, we have stimulated deficient rats with light 5-6 log luminances above their threshold levels, and find that the a-wave evoked is much smaller than the a-wave evoked from normal animals stimulated with light 5-6 log luminances above their threshold. The rapid recovery of the a-wave when vitamin A is fed to moderately deficient animals (fig. 3) is further evidence, I think, that the a-wave is particularly affected in vitamin A deficiency.

In closing, I have nothing to suggest as to why it is that the logarithm of the threshold varies with the visual pigment concentration. However, I would like to point out that Professor Wald has offered a theory (Science, **119**:887, 1954) which might explain such a relation.

THE ROLE OF THE MACULA IN THE ELECTRORETINOGRAM OF MONKEY AND MAN*

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The electroretinogram of the human eye has been shown by the work of Adrian,[1] Armington, Johnson and Riggs,[2] Motokawa and Mita,[3] Auerbach and Burian[4] and others, to consist of at least two portions. One of these, which is slower to develop after stimulation and which reaches its maximum amplitude only after prolonged dark adaptation, has been shown to be a function of the scotopic mechanism of the eye. The other component, faster in its response and reaching its maximum after a shorter period of dark adaptation, is conceived of as due to the photopic system of the retina. Armington has presented evidence, however, that the response may really be a red receptor mechanism.

The hypothetic dual curve of both negative (a-wave) and positive (b-wave) photopic and scotopic components, which construction has been well borne out by numerous studies, was first projected by Armington, Riggs and Johnson[2] and is shown in Figure 1.

The technical approaches toward achieving a record of the human photopic component have been hindered by the fact that the scotopic elements of the ERG are of greater amplitude and are more easily elicited with the recording techniques in use in early clinical electroretinography.

The procedures for "uncovering"—in the words of Granit—the photopic response from the overlying scotopic response have consisted of several techniques:

(1) Spatial differential electroretinography.

Attempts to limit the stimulating light to the area of the macula, where the population of the retinal receptors is primarily cone, have met with little success. The reason for this is that there is both optical and neuronal dispersal of the stimulus so that there can be no delimitation of the stimulated area. This was perhaps best proven by Asher,[5] when he demonstrated that a light directed upon the blind spot produced a most normal ERG.

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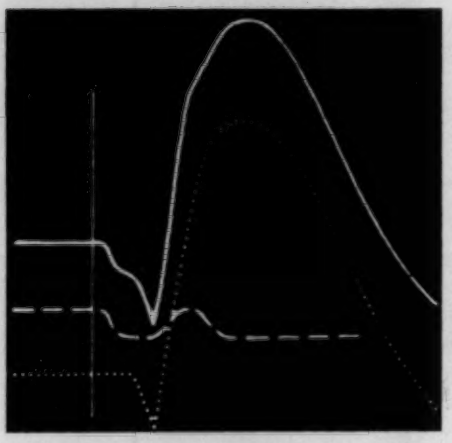

Fig. 1 (Jacobson, et al.). Construction of photopic (dashed line) and scotopic (dotted line) components of the human ERG as described by Armington, Riggs and Johnson.

(2) Attempts to utilize differences in sensitivity of the photopic and scotopic mechanisms to varying parameters of the stimulus.

(a) Temporal-differential electroretinography.

It has been shown by Dodt[6] and others that the photopic response is a much faster one, capable of responding to stimuli of a greater frequency than the scotopic mechanism. By use of a stimulus presented at a frequency higher than the capacity of the scotopic mechanism to follow, one can obtain a measure of photopic functional ability.

(b) Spectro-differential electroretinography.

The photopic mechanism is more sensitive to the long end of the spectrum than is the scotopic mechanism. By use of the stimuli in the red area, therefore, it is possible to emphasize the photopic components.

In addition to the possibility of variation in the characteristics of the stimulus itself, manipulation of the state of adaptation can also serve to emphasize either the photopic or scotopic elements. Recording in the light adapted state will make the photopic response more evident. Armington, and also our laboratory, have found that use of a blue or green background illumination will emphasize the photopic portion of the response to a red stimulus.

The clinical implications of the duality of the response are apparent. The ability to determine, objectively, not only gross retinal function, but to separate photopic from scotopic mechanism functional ability would aid in the determination of the integrity of a retina obscured from ophthalmoscopic view by a cataract. This would be even more significant if it were possible to equate macular function to photopic function.

In studies of patients with macular disease, indeed, there have been a number of reports that the photopic elements of the response are diminished.

Jacobson, Basar and Kornzweig,[7] in 1953, reported a series of 40 patients with far advanced macular disease, in each of whom there was a diminution in response to a deep red stimulus.

Ruedemann and Noell,[8] in 1959, reported a loss of response to a deep red stimulus in patients with central retinal degenerative disease.

Jayle[9] observed an absence of response to red stimuli, a diminished objective flicker fusion frequency and diminution or absence of the photopic a-wave in five patients with severe senile macular degeneration.

Bessiere and Chabot[10] also found a diminished response to red light and an abnormal curve of dark adaptation.

The human macula, although it contains the maximum density of cones—generally accepted to be 147,000 per square millimeter at the fovea—contains about 115,000 cones in the fovea.[11] In the entire macular and perimacular area, including Polyak's area, I, II and III, measuring 5.5 millimeters surrounding and including the fovea, it can be calculated, that there can be no more than 650,000 of the seven million cones of the total retinal population. Thus, it is difficult to equate cone malfunction to loss of photopic response, since only a small proportion of the cones are in the macular area and are

apparently affected by the disease, yet there is a loss of photopic function, as measured by ERG and adaptometry.

The difficulties involved in correlating cone to photopic function and rod to scotopic function, at least in terms of the electroretinogram, have been increased by the recent findings of Tansley.[12] She found that in the pure cone retina of certain squirrels, there is a shift from the typical photopic response to one with scotopic characteristics, upon dark adaptation. Conversely, Davis and Arnott,[13] working with the rabbit, whose retina is predominantly rod, also found a bifid b-wave, similar to that which has been assigned to the photopic and scotopic mechanisms in man.

These two studies indicate that it is possible that under appropriate conditions, the ERG of the rod retina can behave like a photopic receptor and conversely, a cone retina can have scotopic characteristics.

This study was undertaken in an attempt to determine, experimentally, what destruction of the primate macula does to the ERG and to compare the changes to the clinically determined changes in the ERG of patients with macular disease.

Method

The human electroretinogram was recorded using condenser-coupled amplifiers of .033 sec. time constant. Either ink writer of the Grass 111D type were used, or in more recent recordings, cathode ray oscilloscope and camera, to obtain records. The stimulus was provided by a General Radio Strobolux electronic flash unit, whose intensity is approximately 3.2×10^6 candles. The duration of the light source is approximately 10 microseconds. Gelatin filters were used to limit the frequency range of the stimulus. The "red" stimulus referred to was obtained with a filter with a sharp cut off at 610 to 620 millimicrons, as was described in a previous paper.[7]

Recording was carried out within an electrically shielded, grounded chamber, with similar contact lens electrodes of a type previously described[14] used in both animal and human studies. Pupils were fully dilated with 10 percent neosynephrine.

The Meyer-Schwickerath light coagulator was used in a series of five monkeys to produce macular destruction and histologic studies were made to confirm the destruction.

The adaptation curve for patients was obtained with a Goldman-Weekers adaptometer, with fully dilated pupils.

A series of normal patients was examined with the apparatus and a normal range of 125μv. to 350 μv. for b-wave response to the white stimulus and photopic b-wave of 30 to 80 μv. for the red response was obtained. The intensity of the light was diminished for the white stimulus in order to bring the responses within a relatively close range.

Clinical studies

We will present here five patients with different types of macular disease.

Patient 1. (A.M.) A 56-year-old woman (figs. 2, 3, 4, 5) with a history of progressive loss of vision in the right eye for one year. Visual acuity, with best possible correction, is 20/80 in the right eye, 20/20 in the left. The general physical examination reveals nothing remarkable. The eyes reveal incipient cortical cataract in each eye, normal intraocular tension. The fundus of the right eye exhibits normal disc and vessels. The macula shows granular pigmentary disturbance. The fundus of the left eye is normal.

The visual field shows a central scotoma to 1/1000 white test object in the right eye.

The adaptometer revealed a diminished adaptation

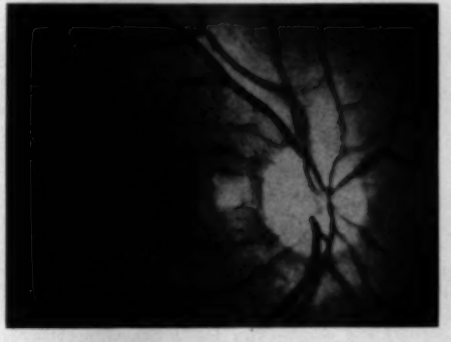

Fig. 2 (Jacobson, et al.). Fundus of patient 1, O.D.

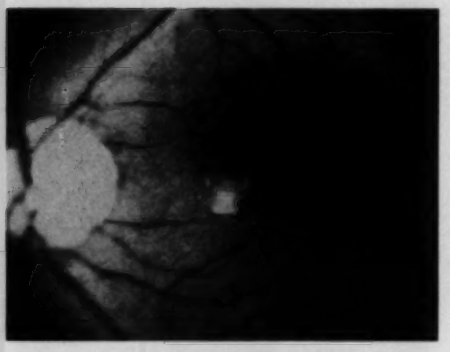

Fig. 3 (Jacobson, et al.). Fundus of patient 1, O.S. (central white area is camera reflex).

in the right eye after 10 minutes, as compared to the left.

The ERG shows an equal response to white stimuli but a markedly diminished x-wave (b_{ph}) in the right eye as compared to the left.

Patient 2. (J.C.) A 10-year-old girl (figs. 6, 7, 8, 9) with a history of progressive loss of vision for two years. There is no history of similar disease in the family, and no known consanguinity.

Visual acuity, with best possible correction, is 20/200 in each eye. General physical examination reveals nothing abnormal. The eyes are normal

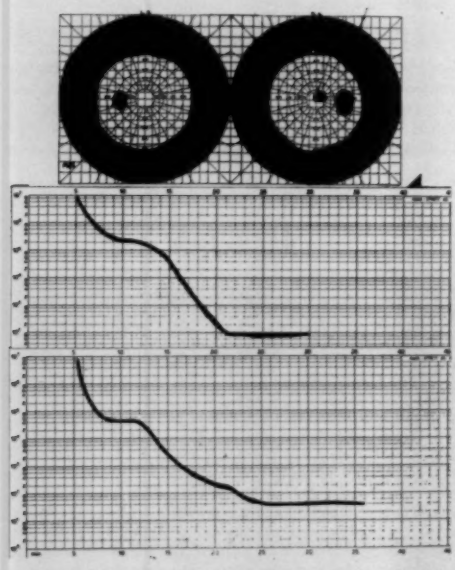

Fig. 4 (Jacobson, et al.). Visual fields (1/1000 white) and adaptometry (O.D. top, O.S. bottom), patient 1.

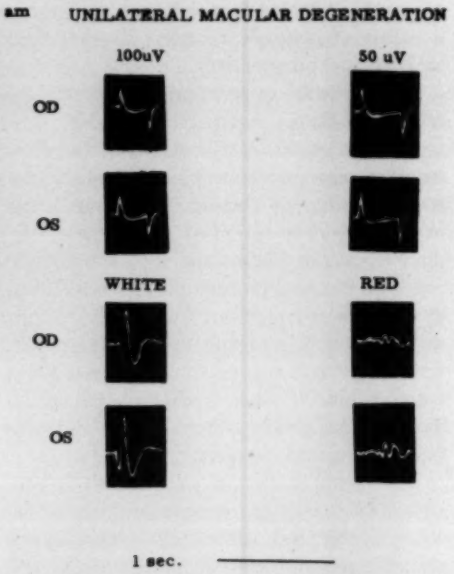

Fig. 5 (Jacobson, et al.). Electroretinogram of patient 1.

except for an elevated discoid lesion at each macula, with evidence of an exudate and hyperemia about the lesion.

The visual field shows a perimacular and partial central scotoma of each eye, with more central loss on the left eye.

The adaptation curve indicates a loss of the cone fraction of the curve.

The ERG shows a diminution of the response to a red stimulus to 18 μv. in the right eye and 20 μv. in the left.

Patient 3. (A.C.) A 52-year-old man (figs. 10,

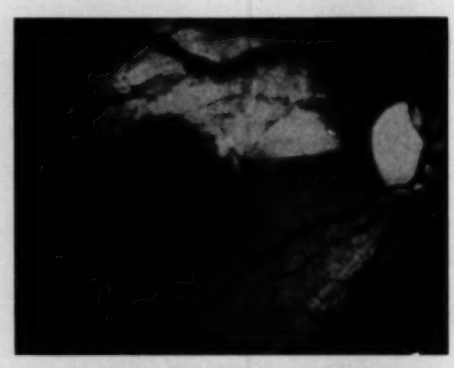

Fig. 6 (Jacobson, et al.). Fundus of patient 2, O.D.

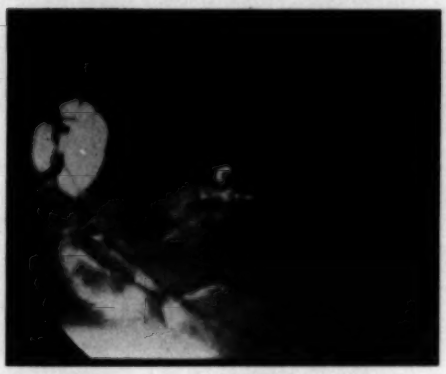

Fig. 7 (Jacobson, et al.). Fundus of patient 2, O.S.

11, 12) with a history of progressive loss of vision, more marked on the left, for seven years.

Visual acuity, with best possible correction, is 20/40 in the right eye, 20/200 in the left. The general physical examination reveals nothing remarkable. There are incipient cataracts in each eye. The intraocular tension is normal. The right fundus reveals arteriosclerotic changes and very minimal disturbance of the pigment about the macula.

In the left eye the optic disc is slightly pale, sclerotic changes of a mild degree are present in

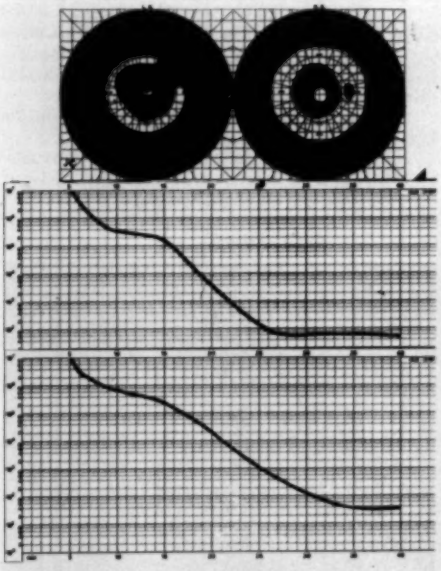

Fig. 8 (Jacobson, et al). Visual fields (1/1000 white) and adaptometry (O.D. top, O.S. bottom), patient 2.

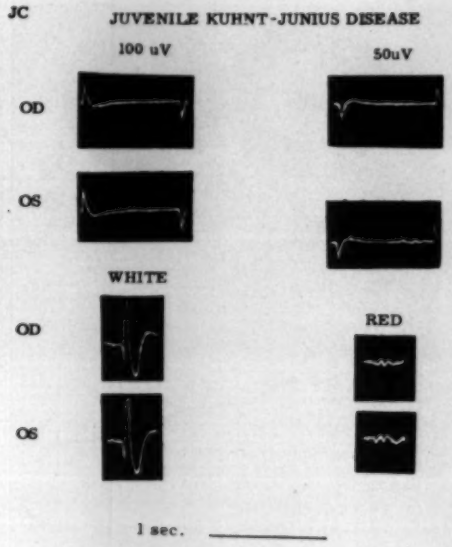

Fig. 9 (Jacobson, et al.). Electroretinogram of patient 2.

the vessels and the macula shows a granular accumulation of pigment, with a slightly depigmented area nearby.

Adaptometry reveals a lesser degree of adaptation in the early portion of the curve in the left eye than the right. There is a central scotoma.

The ERG shows a much smaller photopic b-wave in the left eye than in the right.

Patient 4. (J.F.) A 20-year-old man (figs. 13, 14, 15, 16) with a history of four years of poor vision in both eyes. No family history of significance.

Visual acuity, with best possible correction, is 20/200 in each eye and this is an extramacular

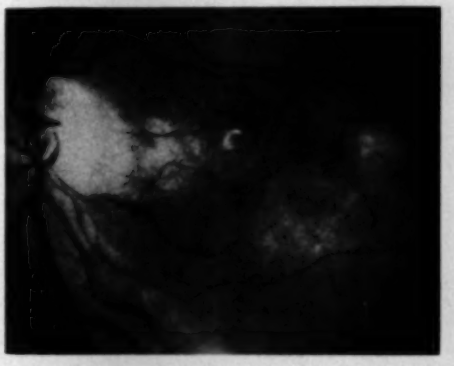

Fig. 10 (Jacobson, et al.). Fundus of patient 3, O.S.

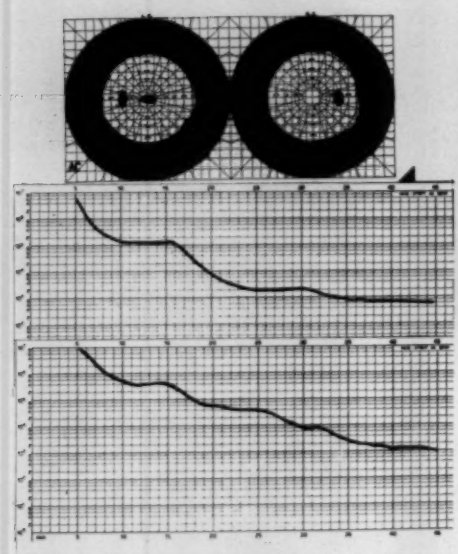

Fig. 11 (Jacobson, et al.). Visual fields and adaptometry (O.D. top, O.S. bottom), patient 3.

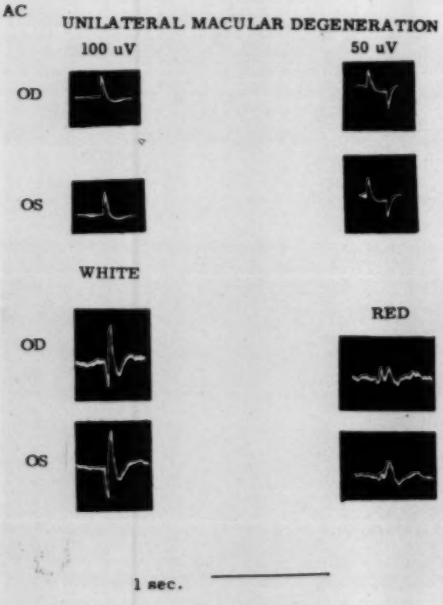

Fig. 12 (Jacobson, et al). Electroretinogram of patient 3.

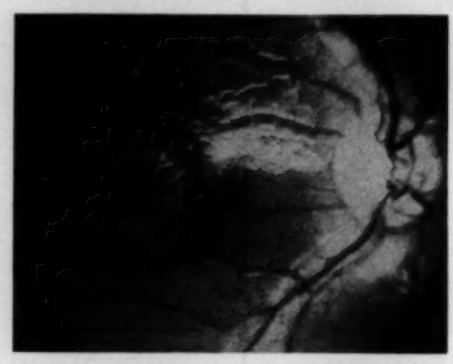

Fig. 13 (Jacobson, et al.). Fundus of patient 4, O.D.

perception. Macular perception is less than 20/800.

The entire physical examination and ocular examination are normal with the exception of the macular area of each eye, at which site the retina seems depressed and atrophic.

The visual field reveals bilateral central scotoma.

The adaptation curve shows a slight diminution in the amplitude of adaptation at 10 minutes.

The ERG reveals an extremely reduced response to a red stimulus in both eyes.

Patient 5. (J.V.) A 19-year-old woman (figs. 17, 18, 19), with a history of loss of vision in the left eye for seven years.

Visual acuity, with best possible correction, 20/20 in the right eye, 20/50 in the left. There is a large pigmented lesion at the posterior pole of the left eye, with the characteristic appearance of a healed chorioretinitis.

The visual field shows a central scotoma in the left eye.

The adaptometry is normal and the ERG is normal. There is a difference between the ERG of the two eyes, the photopic b-wave in the eye with the lesion being reduced to 30 μv., as compared to

Fig. 14 (Jacobson, et al.). Fundus of patient 4, O.S.

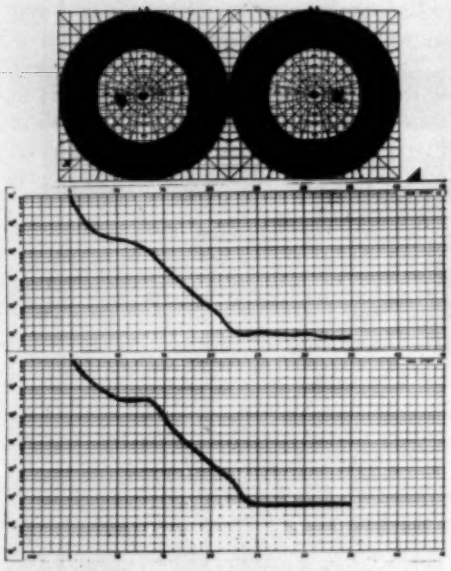

Fig. 15 (Jacobson, et al.). Visual fields and adaptometry, patient 4.

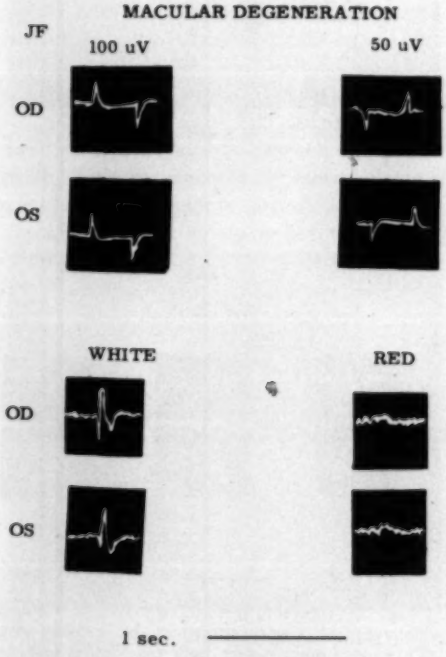

Fig. 16 (Jacobson, et al.). Electroretinogram of patient 4.

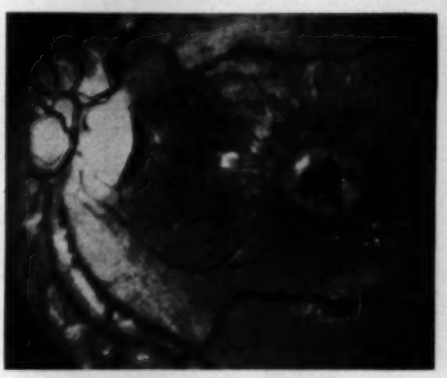

Fig. 17 (Jacobson, et al.). Fundus of patient 5, O.S.

42 for the right eye but both are within the normal range.

These patients as a group, therefore, are all afflicted with retinal lesions which have effectively destroyed or damaged the visual acuity. The degenerative lesions of the macula uniformly show a diminution in the ERG in terms of the response to deep red light. An isolated macular chorioretinitis, on the

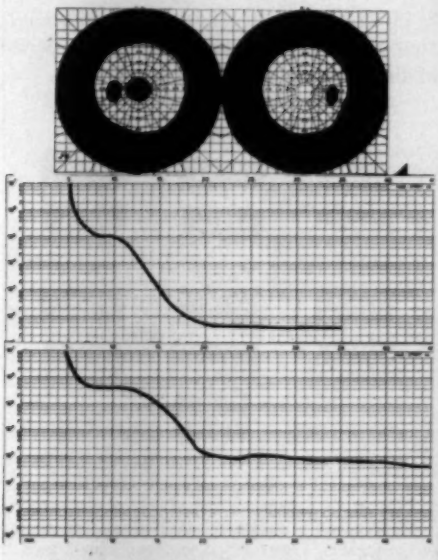

Fig. 18 (Jacobson, et al.). Visual fields and adaptometry, patient 5.

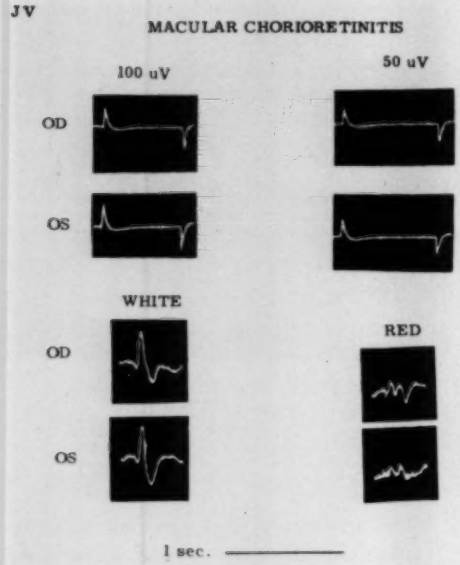

Fig. 19 (Jacobson, et al.). Electroretinogram of patient 5.

other hand, has not affected this function. Adaptometry in the degenerative lesions and to a lesser degree, in the chorioretinitis, tend to indicate a loss of photopic function.

In an attempt to study this phenomenon, four monkeys were subjected to destruction of their maculas by photocoagulation.

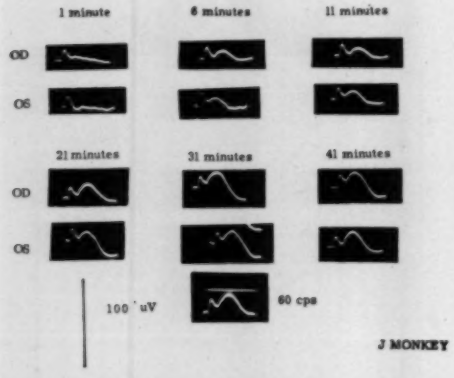

Fig. 20 (Jacobson, et al.). Development of electroretinogram in course of dark adaptation of normal monkey. Times refer to accumulated time in complete darkness.

The results in each case were the same and we will present them as if they were one.

The response of the normal monkey eye to stimulation with the red light changes in the course of dark adaptation, as detailed in Figure 20. The development of the second positive scotopic portion of the response is apparent.

Immediately following light coagulation of the macula of the left eye, in Figure 21, there is an apparent loss of amplitude of the right, coagulated eye in its scotopic portion. The explanation of this we believe, is that the photocoagulator affects the dark adaptation of the coagulated eye in such a manner as to depress the scotopic function. This seems borne out by the return of the scotopic b-wave to normal level, as seen in the record after 21 minutes.

To test this hypothesis, lesions were made in the periphery of the fundus, involving an area approximately equivalent to the area exposed in macular destruction. As shown in Figure 22, the same depression of scotopic function is obtained in the right, coagulated eye (figs. 23, 24, 25).

Apart from this effect, we were able to demonstrate no difference between the coagulated and normal eye of each of the four animals, either in response to single flash ERGs or flickering stimuli, or in response to white or red single or flickering stimuli,

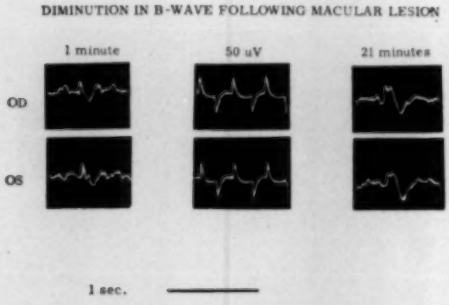

Fig. 21 (Jacobson, et al.). Diminution in scotopic (b-wave) of electroretinogram of monkey eye (O.D.) one minute after light coagulation and recovery by 21 minutes after coagulation.

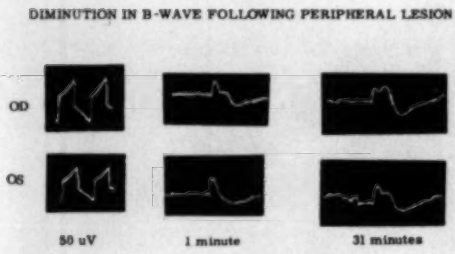

Fig. 22 (Jacobson, et al.). Diminution of scotopic (b-wave) one minute after peripheral coagulation in O.D.; recovery by 31 minutes.

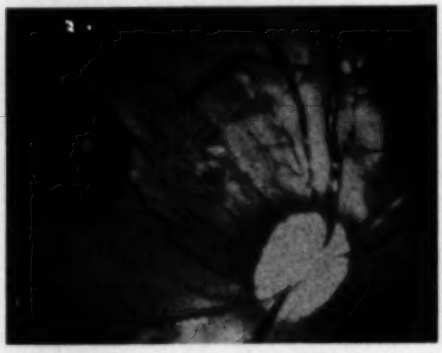

Fig. 23 (Jacobson, et al.). Normal monkey fundus.

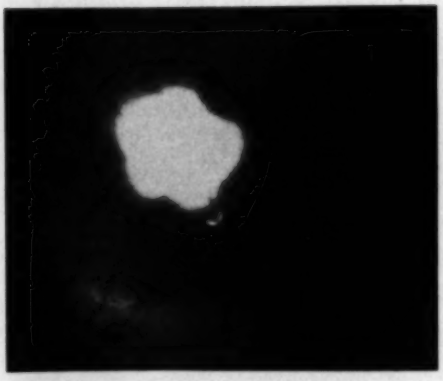

Fig. 24 (Jacobson, et al.). Macular lesion produced in monkey with photocoagulator one hour after exposure.

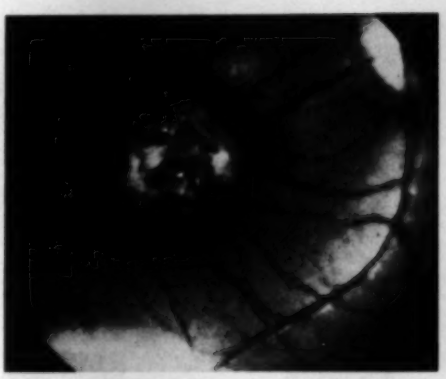

Fig. 25 (Jacobson, et al.). Macular lesion produced in monkey by photocoagulator one week after exposure.

at periods of up to four months post exposure (figs. 26 and 27).

The eyes were removed for histologic study and were examined by Dr. Gerald Kara. His report on one of these eyes, which is typical of all the specimens, follows.

Gross. One area of coagulation can be visualized as a black spot (figs. 28, 29, 30).

Microscopic examination. The macula shows healed chorioretinitis. There is complete destruction of the retinal elements in and around the macular region with glial re-

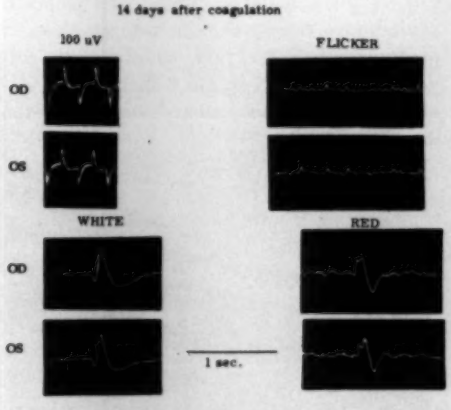

Fig. 26 (Jacobson, et al.). Electroretinograms of right (coagulated) and left (normal) eyes of monkey 14 days after coagulation. All records after 30 minutes dark adaptation.

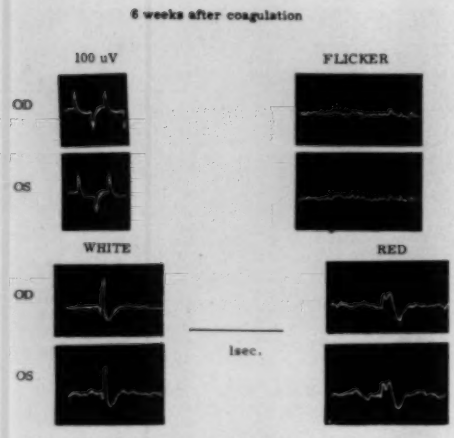

Fig. 27 (Jacobson, et al.). Electroretinograms of right (coagulated) and left (normal) eyes of monkey six weeks after coagulation.

placement between retina and choroid. In this region, is a sheath of connective tissue with pigmentary infiltration simulating a small disciform degeneration of the macular region. The choriocapillaris in this region is destroyed. The area of coagulation is approximately one disc diopter in diameter.

Discussion

The evidence presented in this paper indicates that in monkeys subjected to destruction of the macular area with the photocoagulator we cannot demonstrate any loss of photopic ERG function, as might be evidenced by changes in the flicker fusion frequency, the response to red stimuli, or the

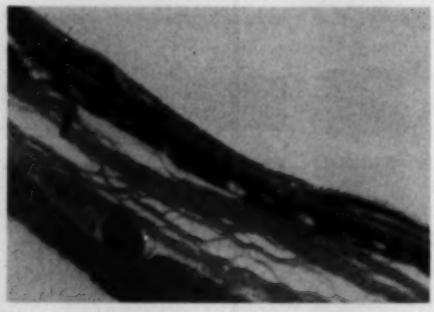

Fig. 29 (Jacobson, et al.). Microphotograph of macular destruction of monkey eye due to photocoagulation.

ERG in the light adapted state.

In contrast to this, there are also presented a number of patients with macular degenerative lesions, in whom there are definite changes in the ERG.

A number of similar reports dealing with clinical ERG changes in macular degeneration are in the literature.

Thus, there is an apparent contradiction between experimental and clinical findings. We believe that the difference is due to the fact that in clinical macular disease states there exists a profound retinal dysfunction, whereas the lesion in the experimental destruction of the macula is more discrete.

A comparison of the cases 4 and 5, both in fairly young individuals, serves to indicate this factor. In the case of patient 5, with unilateral chorioretinitis, there is only a

Fig. 28 (Jacobson, et al.). Normal monkey macula microphotograph.

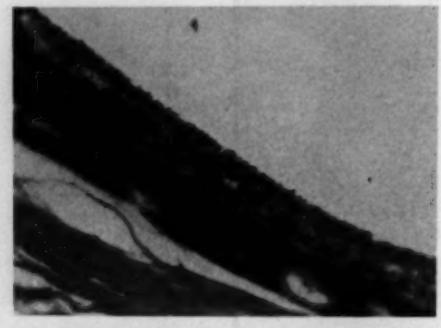

Fig. 30 (Jacobson, et al.). High power of number 29.

localized lesion and the ERG remains almost entirely normal, in spite of a large scotoma.

In the degenerative lesions, however, there is a marked loss of the red response.

The implications for clinical electroretinography of this study are that the techniques for the isolation of the photopic response as a measure of macular integrity—which are of importance in the preoperative analysis of patients with cataract, for example—must be interpreted in light of the fact that discrete retinal and chorioretinal lesions are less likely to be demonstrable than are those of a more chronic, degenerative nature.

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Discussion

Dr. Werner K. Noell (Buffalo, New York): From my point of view, electroretinography is merely a tool of research which we hope will advance our understanding of retinal disease processes. With any such research, as in any other endeavor, one desires specific information, and precision requires that the method of investigation is applied in the best possible way. I see no reason why this would not apply to electroretinography in clinical investigation, and why techniques of recording and measurements are used which in the opinion of many are inadequate and misinforming.

I am referring particularly to the use of equipment such as the electroencephalograph such as you just saw, and to the use of amplifier settings which indicate a time constant of 33 milliseconds, which is much too short.

I am willing to underwrite the conclusions of Dr. Jacobson, but I am sorry to say that I did not get the evidence from his paper.

Dr. Ruedemann last year, in discussing Dr. Jacobson's paper, showed some of our results in central retinal degeneration which were performed at the Kresge Eye Institute with the published technique that is now in use for more than four years, and we have now studied about fifty cases of various types of central retinal degeneration; and in every case in which there is a circumscribed lesion of disc size or larger we find ERG changes which mainly concern to varying degrees the a-wave, the response to flicker, the response to red light, and with regard to various other ERG phenomena.

Dr. Ruedemann and I, like Dr. Jacobson, conclude that the abnormality in retinal function is more extensive in these diseases than indicated by the ophthalmoscopic evidence. May I show a few slides of our material.

[Slide] This is a patient with a familial central pigmented degeneration, with visual acuity in each eye reduced to less than 22/100. The recordings are made by the cathode ray oscilloscope. The flash coincides with the start of the beam, and this is the response to a strong intensity flash in the light. The top lines are the right and the left eye of a normal, and below you see the reduction in this response in the patient.

[Slide] This is a response to a red light stimulus recorded in the same way. The top is the normal

and at the bottom you see the reduction, which involves all components of the response, perhaps more evident in the early phase wave of the typical response to red light.

This is a response in the dark recorded in the patient with higher amplifications. These are calibration marks indicating 200 microvolts. The time from here to here is about 150 milliseconds. You see in the patient a characteristic change in the a-wave. The a-wave emerges slower than in the normal, and is accelerated in its appearance just prior to the b-wave, giving form to these peculiar sharp negative dips from which the b-wave arises.

[Slide] This is also seen in animals. I show you here slides of animals a few days after iodate poisoning. Here you see a similar configuration as in the patient after iodate poisoning, which incidentally affects pigment epithelium and as far as the visual cells are concerned, particularly the outer segments. It is also typical in these animals that the flicker response is much reduced. The waves are smoother, and fusion occurs much earlier.

[Slide] This is a flicker response in the sister of the patient whom we saw before. Here is a tracing of a normal under similar recording conditions, and here the patient had a 20-per-second stimulus.

[Slide] This is from a unilateral disciform degeneration. The left eye, which is represented by this tracing, has 22/100 acuity. The right eye is ophthalmoscopically normal and its vision is normal. I think you can see the marked reduction here in the A wave and a much smoother slope of the B wave.

[Slide] This is the response of this patient to red light. Here again the normal response is shown —that is, the response of the normal right eye, and below is the response of the left eye.

I have shown these slides to indicate that recordings in patients can be made which are comparable to those that we do in the physiological laboratory. I might say also that all this work is done by a trained technician who is capable of studying some four to six patients per day, with a period of examination lasting from half an hour to one hour.

Dr. J. H. Jacobson (closing): I find myself this year, as opposed to exactly the same meeting last year, on the other side of the fence. Whereas last year I presented a paper which discussed the use of the cathode ray oscilloscope and the necessity for it, I find myself in the difficult position of having used an ink writer. This is primarily because some of the clinical records were obtained before we began using the ocsilloscope.

I agree completely (as I did last year) with the necessity (which is why we presented the instrument we did) for the use of the cathode ray oscilloscope. However, since many of the clinical records were obtained with the ink writer, and since it was our intention to try to keep our newer records in conformity with many that are already in existence, they were presented this way. Actually, those recorded in later instances by both techniques, show the same results. In fact the slide of the normal monkey ERG is from oscilloscope records.

I am glad Dr. Noell's results confirm ours. I appreciate his comments. I am at a loss, however, to understand why Dr. Noell says he can not obtain this information from these curves. The information is there.

PERMANENT FRACTIONATION OF THE ELECTRORETINOGRAM BY SODIUM GLUTAMATE*

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Chicago, Illinois

Although various toxic substances have been reported in the past to affect retinal structures, the retinotoxic properties attributed to sodium l-glutamate are unique. Lucas and Newhouse[1] reported in 1957 that administration of sodium l-glutamate to suckling mice caused degeneration of the ganglion cell layer and failure of formation of the inner nuclear layer of the retina, leaving only the receptor cells intact. No information was given on the electrophysiologic behavior of these eyes. We wished to repeat these experiments in order to study the electroretinogram (ERG) of a retina consisting only of receptor cells. Our results are reported below.

Experimental

White mice (Webster Swiss strain)‡ were injected intraperitoneally with a single daily dose of sodium glutamate beginning on the second day of life. The amount injected was increased slightly each day; and injections

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TABLE 1

MOUSE GLUTAMATE DOSAGE

Age (Days)	Dose Mg. per Gm. Body Weight
2	2.2
3	2.5
4	2.8
5	3.2
6	3.4
7	3.6
8	3.8
9	4.0
10	4.2
11	4.4
12	4.6
13	4.8
14	5.0
15	5.2
16	5.4
17	5.6
18	5.8

were continued for from two to 17 days as shown in Table 1. Injection with higher doses caused significant mortality from the procedure.

The eyes of these animals opened on the fourteenth to the sixteenth day after birth. Beginning at the time the eyes opened and at suitable intervals thereafter, the ERG was measured under nembutal anesthesia. Wick electrodes and apparatus were used essentially as described previously by us.[2] Two loops of nickel silver wire appropriately bent and fastened to the supporting board with adhesive tape served at once as speculum and second electrode. At predetermined times an experimental animal and a control animal were killed after measuring the electroretinogram. The eyes were fixed in formalin, imbedded in paraffin, sectioned and stained with hematoxylin and eosin.

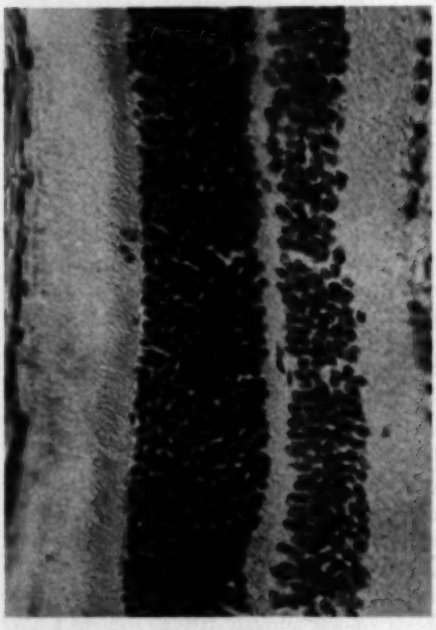

Fig. 1 (Potts, Modrell, Kingsbury). Retina of normal adult mouse. (34 days old)

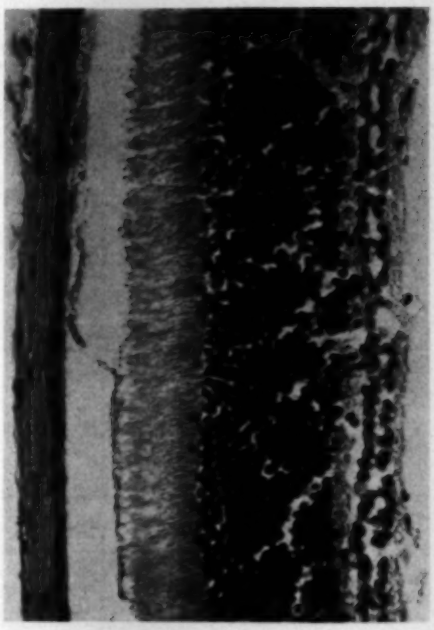

Fig. 2 (Potts, Modrell, Kingsbury). Retina of glutamate treated mouse. (Glutamate 11 days, 54 days old)

In contrast to those of Lucas and Newhouse, our animals were allowed to survive for relatively long periods of time in order to learn how permanent the observed changes in retina and electroretinogram would be.

RESULTS

If injections were continued for seven days or longer, we consistently found the retinal histologic picture reported by Lucas

TABLE 2

THE EFFECT OF GLUTAMATE ON DEVELOPING RETINAL LAYERS OF THE MOUSE

	Normal Controls					Experimental (All animals 30 or more days old when sacrificed)					
Days After Birth	2	4	6	8	10	Retina Mature		Treated with Glutamate for Days:			
						12	34	5	7	9	11
Retinal Layer	Thickness in micra							Thickness in micra			
1) Rod outer segment	—	—	—	—	10	20	30	20	22	22	23
2) Rod inner segment	—	—	—	10	15	20	20	20	20	20	15
3) Outer nuclear layer	20	35	45	55	50	70	70	70	70	70	60
4) Outer reticular layer	—	—	2	10	10	10	10	10	10	10	10
5) Inner nuclear layer		30	65	80	65	80	50	28	15	10	
6) Inner reticular layer	40		20	30	40	60	50	30			
7) Ganglion cell layer		25	30	30	10	10	10	10	15	20	35
8) Nerve fiber layer					15	20	20				

and Newhouse. This consisted of apparently intact receptor cells including the outer nuclear layer. The remainder of the retina was a single or double layer of cells whose nature or function was not ascertainable from the section (figs. 1, 2). Electroretinograms of eyes showing this pattern are uniform in having only an a-wave with no b-wave detectable (fig. 3).

Animals treated for less than seven days showed various degrees of retardation of retinal development. The longer the treatment the more severe the retardation observed. The first portion of Table 2 shows the normal development of the retinal layers in the mouse as measured with an ocular micrometer on paraffin sections. The absolute measurements are subject to the dimensional changes of fixation and imbedding. The relative measurements from layer to layer and from one animal to the next should be valid. The increase in width and the differentiation of each of the retinal layers may be followed to the twelfth day, at which time development is complete. This may be seen by comparison of the 12 day with the 34 day mouse retina. The second portion of Table 2 shows how development of the inner layers is inhibited by glutamate. Even in the animals treated for five days

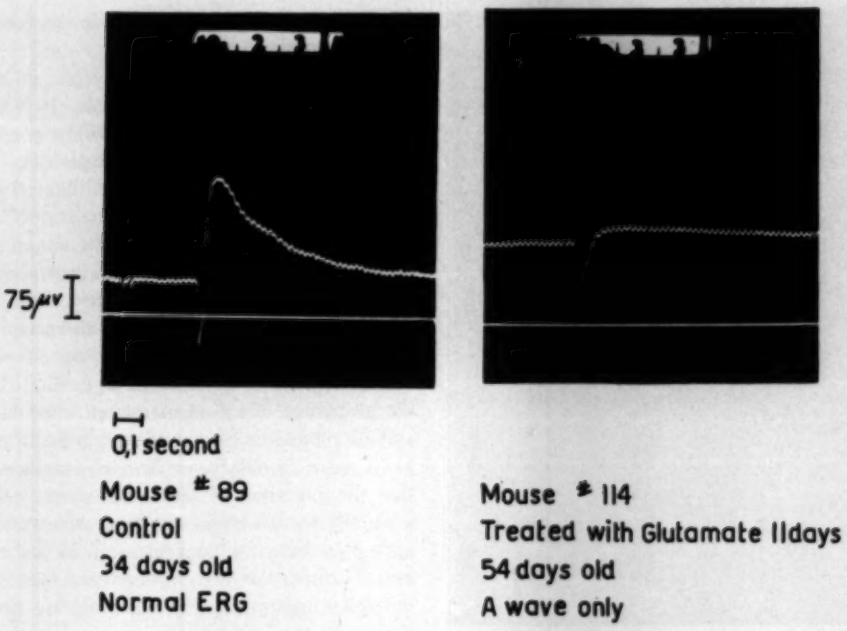

Fig. 3 (Potts, Modrell, Kingsbury). Effect of glutamate on mouse ERG.

where a normal ERG is found, the inner nuclear layer is smaller than normal in width. Figures 4 and 5 show the microscopic appearance and the electroretinographic history of one such animal. Although single examples are presented here, the experiments have been repeated by us many times and the correlation of the histologic picture with ERG is uniform.

Discussion

There appear to be certain inescapable conclusions from the findings presented here. The first of these is that only the initial negative a-wave is connected with the physical presence of the receptor cell layer. For the appearance of the b-wave, the presence of the intermediate cell layers is necessary. It is conceivable, of course, that some portion of the receptor cell responsible for the b-wave is damaged concurrently with bipolar cells but that this damage does not result in a histologically detectable change. There is no experimental evidence to support such an hypothesis.

It is unlikely that the ganglion cells need to be considered in such an analysis, for there is a body of evidence on the integrity of the ERG after optic nerve section and ganglion cell degeneration (for example 3, 4).

There is one previous report which substantiates these conclusions. Noell[3] described the ERG in one eye of a single monkey where the blood supply had been interrupted at the optic nerve and the eye allowed to recover. Degeneration of the inner layers resulted and with this, a negative ERG was observed.

Transient abolition of the b-wave is not infrequently observed following the action of toxic substances on the eye. Granit's analysis of the ERG into three processes was partly based on such a finding.[5] Our earlier studies on the ERG in methanol poisoning[2, 6] showed such an effect with several com-

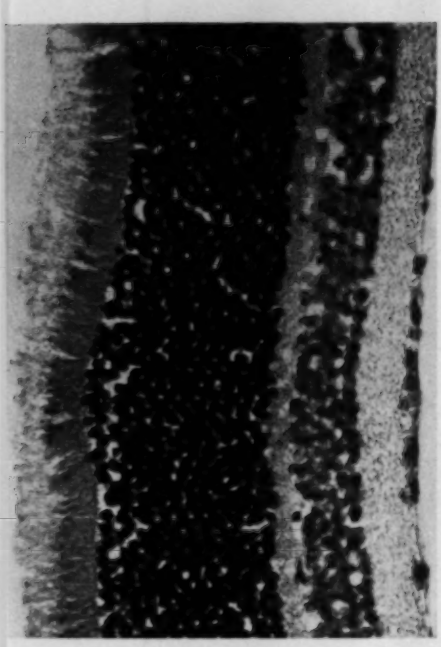

Fig. 4 (Potts, Modrell, Kingsbury). Retina of mouse treated with glutamate five days only. (55 days old)

pounds. It is attractive to hypothesize that these effects represent transient selective inhibition of the bipolar cells while the receptor cells continue to function.

A further question of interest is the mechanism of action of glutamate. It is not an expected physiologic finding that a normal metabolite even in high concentration should have such a destructive effect. The three mechanisms suggested by Lucas and Newhouse all have certain drawbacks voiced by these authors. The suggested indirect effect by adrenal stimulation is unlikely, because other substances such as glycine and p-amino benzoic acid known to stimulate the adrenal do not affect the retina. The suggestion that the glutamate effect on maintenance of high retinal potassium might play a part is unlikely also. It has never been demonstrated that the presence of glutamate which helps maintain normal levels can cause abnormally high potassium ion concentration in the retina. Further, there is no evidence that abnormally high potassium is selectively deleterious to the inner retinal layers. The suggested interference with carbohydrate metabolism is based on the observed depression of anerobic glycolysis by glutamate which is restored by ATP. An uncoupling effect on oxidation and phosphorylation like that of

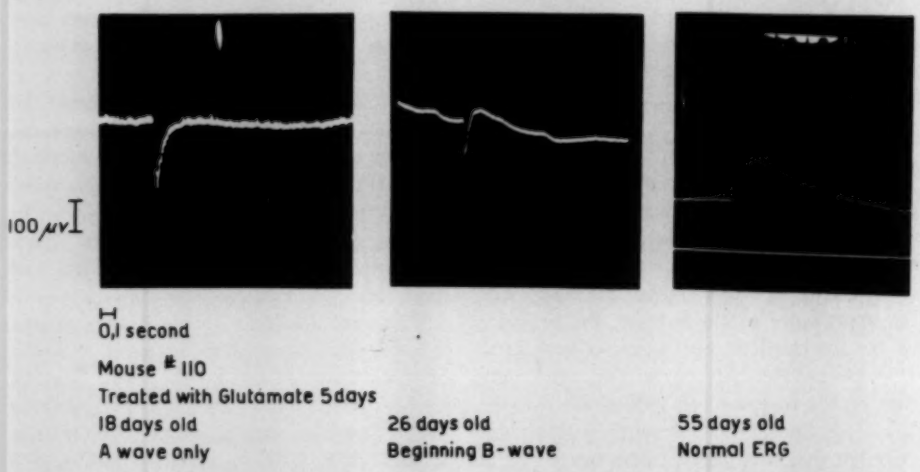

Fig. 5 (Potts, Modrell, Kingsbury). Late recovery of ERG after suboptimal dose of glutamate.

dinitrophenol has also been described. However, dinitrophenol itself will not cause damage to the inner layers of the retina, and ATP will not reverse the glutamate effect.

It seems important to us that the glutamate effect is easily observed only in the developing animal. In the few attempts we made, we were unable to produce the effect in adult mice. In surveying developmental biochemical phenomena, we were impressed by the newer concepts of repression of enzyme formation by products of the specific reaction.[7,8] Several glutamic acid enzymes have been found to increase suddenly in the embryo chick retina at about the time of hatching,[9] and Waelsch has postulated that the glutamate-glutamine system may act as a metabolic buffer.[10] Thus it seems to us that another explanation for the observed effect is most likely—that is: the repression of development of the inner retinal layers by large doses of glutamate given during the period when these layers should be developing. We believe that an enzyme whose reaction product is glutamate (for example glutaminase), is repressed by the high glutamate level. If this enzyme is essential to development of the inner layers because of its participation in a homeostatic system, formation of these layers will be inhibited. If the inhibition is maintained past some critical point, it will be too late for development to occur at all. Experimental investigation of this hypothesis is now under way in the laboratory of one of us (A.M.P.).

Summary

1. Inhibition of the formation of the inner retinal layers in newborn mice by the method of Lucas and Newhouse was repeated by us.

2. Such animals whose retinas are essentially a pure culture of receptor cells show an ERG consisting of a negative a-wave only.

3. In animals receiving inadequate doses of glutamate and showing a normal ERG, the intermediate cell layers are always present.

4. This is considered strong evidence for the origin of the b-wave in the intermediate cell layer.

5. A new hypothesis for the mechanism of glutamate toxicity is proposed.

University of Chicago (37).

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Discussion

Dr. John E. Harris (Minneapolis): This is a particularly easy paper to discuss in certain respects. I have no reason to quibble with the technique. I have no reason to question the results. And it is very fascinating because I can speculate freely as to why glutamic acid is doing this sort of thing. So it has all the attributes of being the perfect paper for a discussant.

As to the interpretation of the electroretinogram, I see no reason to question the conclusion that the beta wave does indeed arise from the bipolar cell layer. I am not particularly knowledgeable in this field, but this suggestion has been made in the past and Dr. Potts' data would seem to confirm it.

I would therefore like to carry on where Dr. Potts left off, and just speculate a bit about the mechanism by which glutamic acid causes these changes in the retina of the developing mouse.

It is a bit surprising to find glutamic acid this toxic. The retina does normally pick up glutamic acid in vitro as shown years ago by Krebs and associates. Indeed these workers found glutamate to be essential for the accumulation of potassium and excretion of sodium by the isolated surviving retina. Whether it plays a similar role in vivo cannot be stated.

Among the possibilities I would like to touch on briefly is the question of the potassium concentration raised by Dr. Potts. I am not thinking here that an excessively high potassium concentration may result from administered glutamic acid but rather the reverse, that the potassium may be depleted from the retina. This is a reasonable extension of some of our studies of the lens. For example, we know that glucose or some metabolic residues are required for potassium uptake by the lens in at least certain circumstances. If the glucose concentration is raised, the potassium uptake and sodium excretion may be blocked. In a similar manner an excess of glutamate might block potassium accummulation in the young mouse retina.

However, I don't necessarily believe this is the mechanism by which the toxic effect of glutamic acid is being exerted. There are two other things I would like to bring out. May I ask the projectionist to show Dr. Potts' last slide, please?

(Slide) The first concerns the question of the toxicity of ammonia itself. The body goes to a great deal of trouble to protect itself from ammonia. Ammonia is quite toxic. The toxicity of hepatic coma for example is considered due to an accumulation of ammonia, and if one floods the system with glutamic acid here (pointing) it would be feasible that we would get an accumulation of an excessive amount of ammonia.

Actually, this does belie in a sense the fact that glutamic acid is used to treat hepatic coma. In this case, of course, glutamic acid is presumably removing ammonia by forming glutamine. However, as Dr. Potts has pointed out, if the young mouse retina contains only a small amount of synthetase, it is quite feasible that ammonia toxicity may result.

The second thing that I think is worth while noting is the possibility that an abnormal protein may be formed. This is known to occur as we deplete the diet of an essential amino acid, for example. Contrariwise if we add an excess amount of one amino acid we can form an abnormal protein. This is something that must be kept in mind.

Moreover, where an abnormal protein is being formed, the effects may not be manifest for quite some period of time. Therefore, I am struck with the possibilities of further studies along this line and the significance that they may ultimately have in our understanding of the group of diseases that we call the abiotrophies. In this group of diseases we assume there is a metabolic fault. This metabolic fault could seemingly come from the formation of an abnormal protein or perhaps an abnormal enzyme.

This is the type of study that I think will lead in the future to a much better understanding of these congenital or developmental conditions which may manifest themselves at birth or may not be apparent for several years.

Dr. Werner Noell (Buffalo): This is very fascinating work, and many people wondered, when the paper by Lucas and Newhouse came out, how specific the effect is. Dr. Potts indicated that he believes this is a direct action of glutamate on the retina. I would like to know what evidence he has in support of it.

Glutamate, as Dr. Potts undoubtedly knows, does not go into the brain and probably also not into the retina if injected intravenously or intraperitoneally in the adult animal. It may possibly go in in the young. In the brain it is very well known that no glutamate goes in. All that goes in is glutamine.

In the adult retina we did experiments with glutamate, injecting very high doses intravenously, and found no acute effects on retinal function, whereas if you get glutamate directly into the retina or directly in the neighborhood of the ganglion cells you see very striking effects, glutamate acting just like potassium chloride. The same happens if one gives glutamate in the other cerebral ventricle. The animals appear to be very rapidly affected in a similar way as with potassium chloride.

With regard to the biochemical slide, I don't think I would call this transaminase, as Dr. Potts indicated, but glutamic acid dehydrogenase—the reaction of alpha-ketoglutarate to glutamic acid. This particular enzyme is known to lag in development in the rabbit during the early stages of life, and later on, between 30 and 60 days, it increases quite remarkably. I personally feel, for instance, that the increase in glutamic acid dehydrogenase in the rabbit retina is very closely related

to the late B wave that one sees during development.

DR. A. M. POTTS (closing): I would like to thank the discussers for their cogent remarks. I think you can see from the discussion that our work is cut out for us as long as we care to study this particular problem.

The question of ammonia toxicity and the formation of an abnormal protein are certainly things that have to be investigated.

Concerning the penetration of glutamate into the retina, one has the evidence of Krebs in vitro that, whatever else glutamate does, it does facilitate the penetration of potassium into the retina. Whether it works at the front door or whether it works inside the cell only in vitro, glutamate does affect an aspect of retinal metabolism. We do plan, however, to do a study with labeled glutamate on animals of the age that show this effect, in order to find out exactly how much goes precisely where.

As far as the slide on mechanism is concerned, this was presented not as a hard and fast hypothesis, as we said, but simply as a possible suggestion. In order to inhibit the synthesis of newly formed enzymes one must be dealing with an enzyme of which the inhibiting substance is the product, not the substrate. Even though the dehydrogenase may very well become involved in time, the fact that it operates on glutamate makes it less likely in our mind to be the specific enzyme that is immediately concerned.

OBSERVATIONS ON EXPERIMENTAL TRACHOMA IN MONKEYS PRODUCED BY STRAINS OF VIRUS PROPAGATED IN YOLK SAC*

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E. JAWETZ, M.D. AND M. OKUMOTO, M.A.
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INTRODUCTION

HISTORY OF EXPERIMENTAL TRACHOMA IN MONKEYS

The literature on the experimental transmission of trachoma to animals was well summarized by Julianelle[1] in 1938, and has been considered in recent studies by Thygeson and Crocker[2] and in the review on ocular virology by Nataf, Bonamour and Lepine.[3] It is certain that the common laboratory animals are refractory to trachoma and that only monkeys and apes contract it. All species experimented with have been susceptible, but the various types of apes (the baboon, chimpanzee, orang-utan, gibbon and so forth) have been more susceptible than the monkeys; the disease induced in the apes has been more severe and inclusion bodies have more often been demonstrable. The experimental disease in all these animals, however, has differed widely from human trachoma; it has always been self-limited and in no instance have pannus or conjunctival scars developed. That it is certainly trachoma, however, has now been established by well-documented human transmission experiments.

The characteristics of experimental trachoma produced by tissue transfer to monkeys include (1) insidious onset with incubation period varying from one to three or more weeks, (2) follicular hypertrophy with relative sparing of the upper tarsus and with the follicles most numerous in the fornices, (3) absence of corneal changes of any kind, (4) scanty exudate, (5) absence of inclusions in epithelial cells, (6) a somewhat inconsistent leucocytic reaction, (7) spontaneous healing after several months, and (8) susceptibility to reinoculation.

OTHER FOLLICULAR DISEASES IN MONKEYS

Inclusion conjunctivitis. So far as is now

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† From the Epidemic Intelligence Service, Communicable Disease Center, U. S. Public Health Service.

known, the only follicular disease which can be produced in monkeys by transmission from man is inclusion conjunctivitis. Experimental inclusion conjunctivitis in monkeys, produced by transfer of tissue or exudate, resembles experimental trachoma in general appearance and clinical course but has generally been a more severe disease with a shorter incubation period and readily demonstrable inclusion bodies. In the baboon, moreover, it very closely resembles the adult form of its human counterpart and in this respect differs sharply from experimental trachoma which bears very little resemblance to trachoma in man.

Spontaneous folliculosis of monkeys. A source of much confusion in trachoma experimentation has been the fact that monkeys may present a noninflammatory follicular hypertrophy known as spontaneous folliculosis. This disease was studied exhaustively by Wilson[4] and Julianelle,[1] and was considered in the more recent report of Thygeson and Crocker.[2] It has been rare in our monkey colony, appearing as one or more raised lymphoid follicles in the fornices, surrounded by normal conjunctiva, unassociated with inflammation or exudate and uninfluenced by mechanical or chemical irritation. Attempts to transmit it from animal to animal by means of scrapings or tissue have failed. In every respect it has seemed to correspond to the transient folliculosis seen in children with hypertrophy of the lymphoid tissue of the nose and throat.

Spontaneous follicular conjunctivitis of monkeys. Recently and in three M. rhesus monkeys only, we have encountered a spontaneous follicular conjunctivitis with inflammation and exudate which could readily be confused, on superficial examination, with experimental trachoma. On cytologic grounds, however, the condition has been sharply differentiable. The exudate has had a high mononuclear cell count but has contained no inclusions, suggesting infection with a virus unrelated to the psittacosis group. Since monkeys have been known to support adenoviruses, there is the possibility that this new disease may be an adenovirus infection. It is now under intensive study, the results of which will be reported in a subsequent communication.

Experimental studies

Experimental trachoma produced by elementary bodies

Since the initial cultivation of trachoma virus in the yolk sac of the developing egg by T'ang et al.,[5] elementary body cultures from various parts of the world have become available for study. In our laboratory at the University of California, six strains of trachoma virus have now been isolated. The characteristics of these strains have been the subject of a recent report.[6] Only the first two strains, however, have been studied intensively on monkeys. The first of these (Bour) was isolated from an early trachoma in a white American adult and the second (Asgh) was isolated from a cicatricial trachoma in a young immigrant from Pakistan who is presumed to have contracted his infection in the Far East. An apparent strain difference has been expressed by a difference in the severity of the experimental disease induced by the two strains in monkeys.

M. cynomolgus and M. rhesus monkeys were available for this study. All monkeys were examined for the presence of spontaneous folliculosis and the few with conjunctival follicles of any type were not used. Each monkey, of a total of 20 cynomolgus and 14 rhesus, was kept in a separate cage and the instruments used in handling the animals and in taking cytologic scrapings were sterilized by flaming between examinations. Inoculations were made with yolk sac material of various dilutions by swabbing the conjunctivas of the upper and lower lids after instilling one drop of dorsacaine solution to induce anesthesia. The technique of swabbing was identical in all instances; the swab, saturated with yolk sac material, was passed first into the upper fornix and the conjunc-

tiva was then stroked with moderate pressure 12 times back and forth between the inner and outer canthi; the same swab was then resaturated, passed into the lower fornix and the conjunctiva stroked another 12 times in the same way. Scarification was not employed.

The animals were examined on the second and third days after inoculation and then reexamined twice weekly so long as there was any observable reaction.

Clinical picture and course. The culture-induced disease has differed from the tissue-transfer disease in the following important respects: it has had (1) a shorter incubation period (two to six days as opposed to seven to 21 days); (2) generally a more acute onset; (3) a greater amount of exudate, particularly during the first weeks of the disease; (4) greater severity, as indicated by the amount of cellular infiltration between the follicles; (5) a higher frequency of papillary hypertrophy of the upper tarsal conjunctiva; (6) inclusions and free elementary and initial bodies in a high percentage of cases; and (7) a consistently specific leucocytic reaction. While the majority of the animals reacting to yolk-sac cultures had acute or subacute symptoms at onset, a minority developed a less severe disease closely resembling the tissue-induced disease (fig. 1). There was in general a direct correlation between the severity of the onset and the titer of the virus inoculum, but Bour virus was able regularly to induce a much more severe disease than Asgh virus. In spite of the greater severity of the culture-induced disease, in no instance did the picture of human trachoma, with its keratitis, pannus and cicatrization, develop. There was no significant difference in duration between the tissue- and the culture-induced diseases; both were self-limited in from six weeks to six months. A few follicles tended to persist after all inflammatory signs had disappeared.

Pathology. Biopsies were taken from selected animals in different stages of the disease and from animals displaying marked differences in the severity of their infections. All specimens examined showed lymph-

Fig. 1 (Thygeson, et al.). Experimental trachoma in M. cynomolgus produced by inoculation with virus (Asgh strain) propagated in yolk sac.

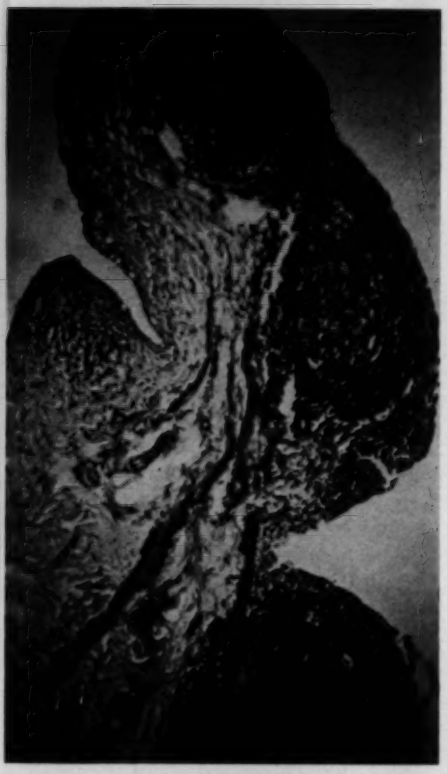


Fig. 2 (Thygeson, et al.). Biopsy from conjunctiva of M. cynomolgus with culture-induced experimental trachoma (Asgh strain) showing follicular hypertrophy in mild form of disease.

oid follicles and cellular infiltration with plasma cells and lymphocytes—the combination that characterizes the pathologic picture of tissue-induced experimental trachoma in these animals. Specimens from mild cases (fig. 2) were in fact just like those from tissue-induced cases and only the specimens from severely involved animals showed more confluent follicle-formation and subconjunctival infiltration (fig. 3) than ever has been seen by any of us in tissue-induced cases. In no instance did we encounter any cellular necrosis or scar formation such as is seen in human trachoma.

While "spontaneous folliculosis," in which there is no cellular infiltration, was readily differentiable from the culture-induced experimental trachoma, whether mild or severe, the one biopsy from the "spontaneous follicular conjunctivitis" previously described revealed follicles and infiltration (fig. 4) such as are seen in mild forms of experimental trachoma.

Cytology of exudate smears and epithelial scrapings. In the report of Thygeson and Crocker,[2] consideration was given to the diagnostic value of the study of conjunctival scrapings from experimental trachoma. Although neither trachoma inclusions nor free elementary bodies were ever encountered in this tissue-induced disease, the active infection was frequently, though not always, associated with an abundance of neutrophils and occasionally with macrophages (Leber cells) and plasma cells. In this respect it resembled human trachoma, and as in trachoma there was a clear correlation between the severity of the inflammatory signs of the disease and the percentage of neutrophils in the exudate; as the disease subsided, the percentage of mononuclear cells rose. It is a valuable difference that in spontaneous folliculosis, which is a noninflammatory disease, a leucocytic reaction never has been observed.

In culture-induced experimental trachoma, the diagnostic value of examining epithelial scrapings has been greatly enhanced by the

Fig. 3 (Thygeson, et al.). Biopsy from conjunctiva of M. cynomolgus with culture-induced experimental trachoma of acute onset (Bour strain) showing severe cellular infiltration.

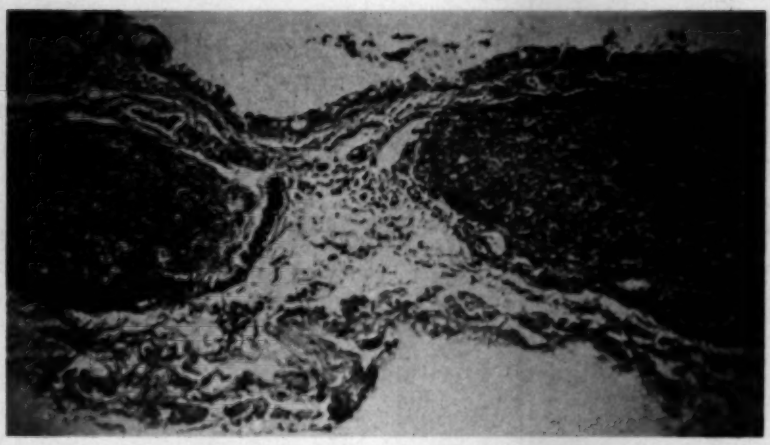


Fig. 4 (Thygeson, et al.). Biopsy from conjunctiva of M. rhesus with "spontaneous follicular conjunctivitis" showing follicular hyperthrophy.

finding of typical cytoplasmic inclusions. These inclusions have corresponded in every way to those found in human trachoma; the same elementary and initial body stages (fig. 5) have been in evidence and the same iodine-staining carbohydrate matrix (fig. 6). In the more severe cases of the culture-induced disease, particularly in its early stages, free elementary and initial bodies have been seen, sometimes in extraordinary abundance and corresponding in this respect to their abundance in the rare acute cases of human trachoma. Inclusions have appeared in scrapings from all parts of the conjunctiva and in a few instances even from the cornea (fig. 7) despite the absence of clinical corneal disease.

In addition to the inclusion body findings, a neutrophilic exudate has regularly characterized the culture-induced disease. In cases with much inflammation the exudate has been almost entirely neutrophilic, but as the inflammation has declined, the percentage of mononuclear cells has increased. Neutrophils have always been present in the active stages, however. In addition, plasma cells and macrophages (fig. 8) have been seen more regularly than the tissue-induced disease. The diagnostic value of plasma cells and macrophages in human trachoma has been fully discussed in a report by Thygeson.[7] These cells were not observed by us in scrapings from spontaneous folliculosis.

The diagnostic value of these exudate findings has been further augmented by our demonstration of a purely mononuclear-cell exudate in the three rhesus monkeys with the "spontaneous follicular conjunctivitis" described above. There remains experimental inclusion conjunctivitis from which there appears at the moment to be no cytologic means of differentiating experimental trachoma.

Reactivation with steroids. In 1952 Ormsby and associates[8] called attention to the reactivating effect of topical steroids on subsiding inclusion conjunctivitis, and later Nataf and Freyche[9] noted a similar effect on trachoma. This cortisone effect has been studied by other observes[10] throughout the world and has been advocated as a provocative test of cure in old cicatricial cases. Thygeson and Crocker[2] obtained mild clinical reactivation in only three of 12 animals as a result of twice-weekly subconjunctival injections of cortisone or hydrocortisone, but were unable to demonstrate inclusions in the reactivated cases.

Greater success has been achieved in subsiding inclusion-negative culture-induced

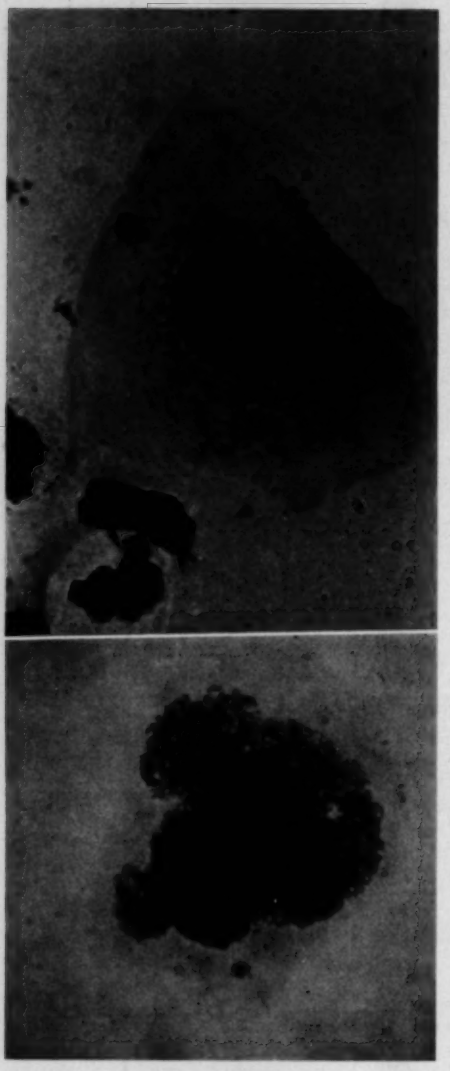


Fig. 5 (Thygeson, et al.). Inclusion bodies in conjunctival epithelial cells from culture-induced experimental trachoma (M. cynomolgus).

disease, clear-cut clinical reactivation having been effected in four cynomolgus monkeys by subconjunctival injections of 0.25 cc. meticortelone over periods ranging from 25 to 44 days. In two of these animals inclusions reappeared (fig. 9) and in all four there was an increase in the typically neutrophilic exudate. In every instance the reactivated disease subsided rapidly when the steroids were withdrawn.

The monkey as an experimental animal in trachoma

Although some workers, notably Julianelle considered the monkey to be a reasonably reliable experimental animal in trachoma, others, like Wilson for example, cautioned against confusing experimental trachoma with spontaneous folliculosis. Thygeson and Crocker[2] concluded that monkeys and baboons could be used with profit in the study of inclusion conjunctivitis, but that conclusions based on monkey studies in trachoma must be confirmed by human inoculation experiments because of the lack of pannus and scarring in the monkey disease.

This report presents a much more favorable case for the usefulness of the monkey in tests with yolk-sac cultures of trachoma virus. In our small series no example of natural immunity has been encountered and it appears that the monkey may be at least as sensitive as the yolk sac to trachoma virus. The reliability of diagnosing the experimental disease and of differentiating it from spontaneous follicular disease in monkeys has been much improved by the demonstration of inclusion bodies, free elementary and initial bodies and a characteristic leucocytic reaction. In ruling out spontaneous disease, however, caution must still be exercised, clinical observations requiring confirmatory cytologic observations.

The following are several examples of the successful use of monkeys in a study of differences between our Bour and Asgh strains.

Relative infectivity of Bour and Asgh strains of trachoma virus. To study the relative infectivity of the two strains of virus, conjunctival inoculations were made with progressively more dilute suspensions of trachoma virus. The cynomolgus monkeys were infected with material from several different egg passages, but the inocula used for rhesus

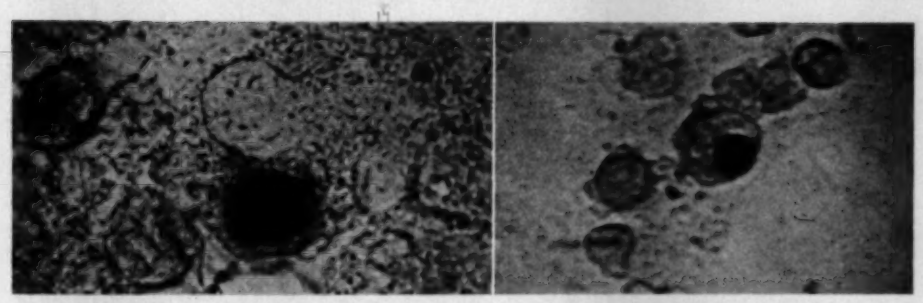


Fig. 6 (Thygeson, et al.). Iodine-stained carbohydrate matrix of inclusion body from culture-induced experimental trachoma (M. cynomolgus).

monkeys were made up of dilutions of one pool each of the Asgh and Bour strains.

The disease produced in the monkeys' eyes by more concentrated material was usually so florid that there was no problem in diagnosing a definite clinical take. With lower doses of virus, however, the clinical manifestations were much less marked and it became difficult to decide what should be accepted as a satisfactory criterion of infection. When the two virus strains were compared in the same species of monkey, it was apparent that fewer egg-lethal doses of the Bour strain were needed to produce a more severe disease in both cynomolgus and rhesus monkeys. At all dilutions the Bour strain was associated with a more severe disease than the Asgh strain. It is possible that the rhesus monkey is somewhat more resistant to infection than the cynomolgus but no conclusion could be drawn from the small number of animals used.

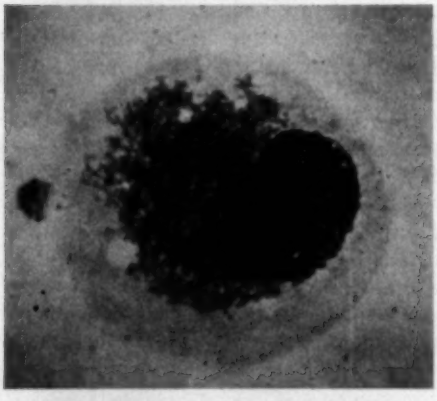


Fig. 7 (Thygeson, et al.). Inclusion body in corneal epithelial cell from experimental trachoma of acute onset (M. cynomolgus).

Certain parameters of the infection are tabulated in Tables 1, 2, 3, and 4 to demonstrate the differences between the dilutions of the two virus strains. The infectivity of the Bour strain for cynomolgus monkeys is summarized in Table 1. At all dilutions a severe follicular conjunctivitis appeared after inoculation, but lower doses resulted in a longer incubation period and a later day of maximum intensity. Inclusions were always demonstrable in the conjunctival scrapings. Similar results were achieved in rhesus monkeys over an even greater range of dilutions of Bour virus (Table 2). All monkeys in this group became clinically infected. Lower dilutions were associated

TABLE 1

INFECTIVITY OF CULTIVATED TRACHOMA VIRUS FOR CYNOMOLGUS MONKEYS BOUR STRAIN

Inoculum, egg-lethal doses/ml.*	$10^{6.3-6.7}$	$10^{5.3-5.7}$	$10^{3.4}$
Clinical infection, no. infected/no. inoculated	4/4	5/5	1/1
Incubation period (day after inoculation)	3.5	6.2	6
Day of maximum clinical disease	12	10.3	21
Inclusion-positive smears	4/4	5/5	1/1

* Negative log of dilution resulting in 50 percent death of eggs.

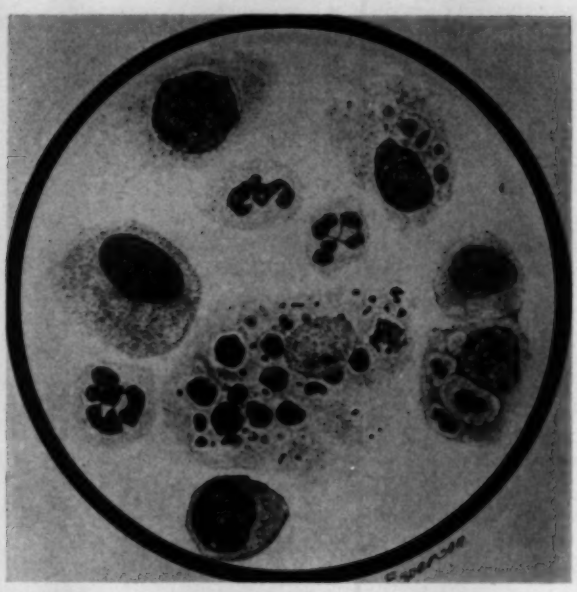
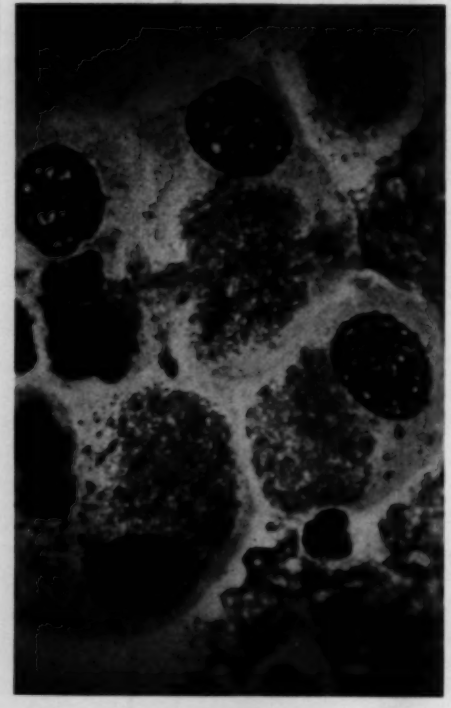


Fig. 8 (Thygeson, et al.). Epithelial scrapings from culture-induced experimental trachoma of M. cynomolgus showing Leber cells and neutrophils. Drawn from a magnification of 1000 ×.



with a later day of maximum clinical disease, but the incubation period remained the same over a 100-fold range of concentration. Inclusion-positive smears were found in all rhesus monkeys except for one of those receiving the lowest dose.

With the Asgh strain of virus the disease was produced less regularly and was clinically less severe when present. In cynomolgus monkeys (Table 3) the onset was invariably insidious. Only at the highest concentrations of virus was it possible to infect all the cynomolgus monkeys, and in two instances they failed to respond to $10^{5.4}$ and $10^{4.4}$ egg-lethal doses. Inclusion-positive smears appeared only after the highest doses. Four dilutions of the Asgh strain were given to a group of rhesus monkeys (Table 4) and all these animals manifested some degree of clinical disease. The incubation period was longer and the day of maximum

Fig. 9 (Thygeson, et al.). Multiple inclusions in epithelial scrapings from steroid-reactivated experimental trachoma.

TABLE 2

INFECTIVITY OF CULTIVATED TRACHOMA VIRUS FOR RHESUS MONKEYS BOUR STRAIN

Inoculum, egg-lethal doses/ml.*	$10^{4.4}$	$10^{3.4}$	$10^{2.4}$	$10^{1.4}$
Clinical infection, no. infected/no. inoculated	1/1	1/1	2/2	2/2
Incubation period (day after inoculation)	2	6	6	6
Day of maximum clinical disease	6	16	16	22
Inclusion-positive smears	1/1	1/1	2/2	1/2

* Negative log of dilution resulting in 50 percent death of eggs.

clinical disease later as the dilutions were lowered. It was possible to find inclusions in only one monkey at each of the two highest titers and none in the four monkeys receiving less virus.

Reinoculation with yolk-sac virus after recovery from experimental trachoma. Table 5 summarizes the results of reinoculation of five cynomolgus monkeys that had recovered from infection with Asgh virus. Second inoculations were made with Bour strain in four instances and with Asgh in a fifth. The second disease was comparable to primary infections with the same virus in respect to severity, incubation period and presence of inclusions. This would indicate that the reuse of animals after natural or drug-induced recovery from experimental trachoma would be a safe experimental procedure.

TABLE 3

INFECTIVITY OF CULTIVATED TRACHOMA VIRUS FOR CYNOMOLGUS MONKEYS ASGH STRAIN

Inoculum, egg-lethal doses/ml.*	$10^{5.3-6.4}$	$10^{4.4}$	$10^{3.4}$
Clinical infection, no. infected/no. inoculated	6/6	2/3	0/1
Incubation period (days after inoculation)	4	6	—
Day of maximum clinical disease	14.5	20	—
Inclusion-positive smears	4/6	0/3	0/1

* Negative log of dilution resulting in 50 percent death of eggs.

TABLE 4

INFECTIVITY OF CULTIVATED TRACHOMA VIRUS FOR RHESUS MONKEYS ASGH STRAIN

Inoculum, egg-lethal doses/ml.*	$10^{5.4}$	$10^{4.4}$	$10^{3.4}$	$10^{2.4}$
Clinical infection, no. infected/no inoculated	2/2	2/2	2/2	2/2
Incubation period (days after inoculation)	3	3	4.5	4.5
Day of maximum clinical disease	4.5	8	6.5	10
Inclusion-positive smears	1/2	1/2	0/2	0/2

* Negative log of dilution resulting in 50 percent death of eggs.

DISCUSSION

The monkey as an experimental instrument in studies of trachoma leaves much to be desired. The animal is expensive to procure and to maintain and is peculiarly subject to pneumonitis and to diarrheal diseases that carry a high mortality rate. The experimental disease in the monkey produced by tissue transfer usually begins insidiously after a long incubation period, runs a mild course and is sometimes difficult to differentiate clinically from spontaneous follicular disease. Nevertheless, the monkey is the only animal available and in view of the potential severity of the human disease, the use of human volunteers must be sharply limited at best. Every effort must therefore be made to improve the reliability of monkey experiments.

TABLE 5

REINOCULATION OF PREVIOUSLY INFECTED MONKEYS WITH CULTIVATED TRACHOMA VIRUS

	First Inoculation	*Second Inoculation*	
Strain:	Asgh	Bour (4)	Asgh (1)
Egg-lethal doses/ml.*	$10^{5.3-6.4}$	$10^{4.3-6.4}$	$10^{6.4}$
Incubation period (days)	3	3.8	3
Inclusion-positive smears	5/5	4/4	1/1

* Negative log of dilution resulting in 50 percent death of eggs.

In this present study we have shown that M. rhesus and M. cynomolgus monkeys are susceptible to infection with virus grown in the yolk sac and that this susceptibility appears to be at least as great as the susceptibility of the yolk sac itself. We have shown that in infections of acute or subacute onset, the clinical disease is readily differentiable from the natural follicular disease of these animals, but that in mild infections with insidious onset great care must be exercised in diagnosing experimental trachoma. Fortunately even in mild cases the cytologic diagnosis has proved to be reliable. It should be pointed out, however, that at the moment we have no means of differentiating experimental trachoma from experimental inclusion conjunctivitis in the monkey, since both display the same conjunctival changes and cytology.

It is of interest that acute trachomatous infection in monkeys has not been accompanied by keratitis or cicatrizing changes in the conjunctiva, in spite of intense cellular infiltration and numerous inclusions. Inclusions have even been seen in scrapings from the cornea during the acute phase of the disease in spite of total lack of clinical evidence of corneal disease. Nor has the distribution of the follicular hypertrophy in these animals resembled its distribution in human trachoma. The upper tarsal conjunctiva, often the site of the first and heaviest involvement in man, is the last area to be involved in the monkey.

The use of monkeys to titrate egg-cultivated trachoma virus has been limited by the lack of a satisfactory end point. Apparently the susceptibility of the monkey is such that infections are induced occasionally even with extremly dilute yolk sac material. If a large number of monkeys could be used for each dilution of virus, it would be feasible to make valid statistical comparisons similar to titrations in embryonated eggs where the 50 percent lethal dose is taken as an end point. Since the monkey is an expensive animal, both to buy and to maintain, consideration should be given to possible end points other than clinical conjunctivitis, for example, (1) disappearance of the characteristic cytologic picture, (2) disappearance of inclusion bodies,(3) lack of acute or subacute onset, and (4) incubation periods longer than an arbitrary maximum. Although the conjunctival cytology is an extremely useful qualitative guide, it is difficult to compare smears quantitatively. Inclusion bodies in smears can be regarded as uniquely specific evidence of infection, but their demonstration depends on the number of smears taken and the time spent in examining them. The difference between an acute and an insidious onset is no problem, but the borderline between a subacute and an insidious onset is sometimes difficult to determine. The use of the incubation period as a criterion for titrations can be criticized, but it should be possible arbitrarily to set a limit to the time during which manifestations of infection should appear; animals not responding to inoculation within this time limit would be considered negative even if they should develop disease later. This would obviate the necessity of following the clinical disease for an undue length of time, and the monkeys could be treated with antibiotics and used again without undue delay.

There are apparently two types of "spontaneous" follicular disease in monkeys. The first is the well-known spontaneous folliculosis which has been well described in the literature, particularly by Wilson.[4] It resembles folliculosis in children and probably represents an adenoid hypertrophy of the conjunctiva. In our experience this disease has not been transmissible from monkey to monkey and has not been affected by mechanical or chemical irritants. The second type of follicular disease is definitely a follicular conjunctivitis with inflammation and exudate. The mononuclear exudate certainly suggests viral infection, although to date our studies have not provided a definite answer. These studies are continuing. One wonders if this disease may not be the one mistaken

for trachoma by Noguchi in the course of his etiologic investigations of the disease.

Summary and Conclusions

1. Experimental trachoma in monkeys can be produced regularly by inoculating them with yolk-sac cultures of elementary bodies obtained from trachoma cases. No example of complete natural resistance has been found.

2. The experimental disease differs from that produced by tissue transfer in having a shorter incubation period, an acute or subacute onset and inclusion bodies in epithelial scrapings.

3. The cytologic findings in culture-produced experimental trachoma resemble the findings in human trachoma and the cytologic picture is regarded as having diagnostic significance. It is particularly valuable in the recognition of early and mild infections and in the differentiation of experimental trachoma from "spontaneous folliculosis" and "spontaneous follicular conjunctivitis."

4. The experimental disease produced by culture, even when its onset is acute, is not accompanied by pannus, cicatrization, or major involvement of the upper tarsus and in these respects differs sharply from human trachoma.

5. The usefulness of the monkey as an experimental animal in the study of trachoma seems to have been established; by its use it has been possible to perform valid virus titrations and to make strain comparisons.

University of California, School of Medicine (22)

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Discussion

Dr. Alson E. Braley (Iowa City, Iowa): Dr. Thygeson's paper has pointed out that the monkey can be used as a test animal to aid in the diagnosis and study of trachoma. It has been estimated by Grayston et al that trachoma afflicts over 400 million people in the world, and that it is the world's leading cause of blindness. It is therefore important to have a good animal or culture medium to help make the diagnosis of trachoma.

Dr. Thygeson has pointed out that the monkey is as sensitive a test animal as is the yolk sac.

The World Health Organization's Expert Committee on Trachoma insists that the final proof that a virus under cultivation is the cause of trachoma should be the production of typical trachoma in human volunteers after sufficient passage in culture to eliminate by dilution any virus of the original inoculation.

Dr. Thygeson has shown here that the monkey can be used, provided the clinical course and the cytology of the conjunctiva are used for trachoma diagnosis in monkeys. The absence of keratitis in monkeys is disturbing, but for a clinical evaluation of the disease the inclusions have been found in scrapings from the corneal epithelium.

In discussion of the host range of trachoma virus

grown in yolk sac, Grayston et al say that the monkey conjunctiva is the only tissue of any laboratory animal that may be infected. Many years ago I pointed out that the monkey cervix can also be infected with trachoma virus. I wonder if Dr. Thygeson would comment on the use of the monkey cervix as a test tissue.

Dr. Thygeson feels that monkey trachoma is a disease that can be recognized, and that human volunteers are not essential to prove the diagnosis. There is a remarkable difference between human trachoma and monkey trachoma. I wonder if Dr. Thygeson would care to discuss some of the reasons for the marked difference. Is there any reason why, when the virus is present in the conjunctival epithelium, there are still no signs of inflammation in the cornea?

I would like to ask Dr. Thygeson what is the most useful diagnostic aid in trachoma. I think we can include the laboratory aids with the clinical diagnosis—such as smears for inclusion bodies, isolation in yolk sac, inoculation of monkey conjunctiva, and possibly complement fixing antibody titer in the blood. The latter has been most unreliable and erratic.

It has been a pleasure to read Dr. Thygeson's paper, and I would like to add that, from my own experience, while monkey trachoma is not like human trachoma, it is a disease that is so different in the monkey that it should be called monkey trachoma. The clinical picture combined with the cytological picture indicates that the monkey conjunctiva can be used as a test tissue instead of human volunteers.

Because of the spontaneous appearance of a not-unlike Beale's type of conjunctivitis, I wonder if Dr. Thygeson has also had any experience with the susceptibility of the monkey conjunctiva to adenovirus.

I want to thank Dr. Thygeson for the opportunity to read his paper, and also for the opportunity to discuss it.

DR. PHILLIPS THYGESON (closing): I thank Dr. Braley for his interesting discussion. A number of points he raised are of importance. I am thinking particularly of his demonstration some years ago of the susceptibility of the cervix of the baboon to trachoma virus. That has become particularly important in view of the present controversy as to whether inclusion conjunctivitis and trachoma are the same disease or whether they are distinct as I have always maintained and as epidemiologic findings certainly indicate.

We have just isolated a single strain of inclusion blennorrhea virus, and we have made only three monkey inoculations so far. The experimental disease in the monkey with inclusion conjunctivitis has not been distinguishable from that of trachoma, so I feel the monkey is not going to help us greatly. We will have to use human inoculations to make a distinction between the two diseases unless clear cut biochemical or immunologic differences can be shown between the two viruses.

I regret that Dr. Braley's demonstration of cervical inoculation with trachoma materials has been lost in the literature, but I will make every effort to revive it. We are going into the venereal aspects of inclusion blennorrhea again. Definitely this is an important problem.

In regard to adenoviruses in the monkey, several strains of these viruses have been recovered from the monkey—monkey feces in one instance, monkey kidney tissue in another—but all attempts to produce an infection with Type 8 adenovirus, the type in which we have been most interested, have failed with us. However, the spontaneous follicular disease of rhesus monkeys which we described looks like an adenovirus type and has the same mononuclear cellular reaction. We now have this disease under study. I am almost positive that this is the disease that Noguchi encountered with his Bacterium granulosis work, and I believe we had it going at one time, too. It is a disease which has been transmissible indefinitely from monkey to monkey, unlike experimental trachoma which dies out after three, four or five passages. I believe we can keep this disease going indefinitely.

In advocating the monkey as an experimental animal, I might say that it has no conceivable use at the moment in any survey work; even though the egg is possibly not as sensitive, it is much cheaper and more convenient. For certain titrations, determination of strain differences, and so on, I think the monkey can be used. Definitely apes are more sensitive, but the monkey is the only practical laboratory animal available to us to use in this work.

THE EFFECT OF CORTICOSTEROIDS ON EXPERIMENTAL OCULAR TOXOPLASMOSIS*

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The therapy of Toxoplasma uveitis has been a subject of some uncertainty in recent years. As early as 1952, Eyles[1] and independently Summers[2] discovered that pyrimethamine (Daraprim) was of therapeutic benefit in acute experimental toxoplasmosis. In animals, sulfonamides were found to potentiate the toxoplasmacidal action of pyrimethamine[3] and an extensive clinical trial by Ryan[4] and his coworkers indicated this combination of medicines to be beneficial in the treatment of posterior uveitis. Perkins,[5] in a double-blind trial of pyrimethamine and sulfonamides found that when these drugs were given to patients with positive Toxoplasma dye tests greater improvement was observed than when placebos were administered. Furthermore, if all patients with posterior uveitis were treated, improvement was greater in the group with positive dye tests. There is evidence, however, that hypersensitivity may be important in chronic ocular toxoplasmosis. It has been the experience of some excellent observers, such as Hogan,[6,7] that there are cases of posterior uveitis which are almost certainly caused by toxoplasmosis in which the course does not appear to be altered by specific drug therapy; Frenkel[8] and Jacobs[9] have postulated that if cysts were to rupture in cases of chronic ocular toxoplasmosis, hypersensitivity might be an important factor in the ocular disease.

Furthermore, previous workers have demonstrated that when nonliving antigens, such as bovine albumin, are inoculated into the vitreous a reaction occurs after about a week, which spontaneously subsides. For some weeks thereafter, however, recurrence of ocular inflammation can be brought about by systemic challenge of the animal with the same antigen. It appears as if the recurrences of uveitis can be induced on an allergic basis as long as sufficient antigen remains in the vitreous.

Toxoplasmas presumably act as antigens in a similar manner; however, these organisms form dormant forms and cysts. Once present in the eye, therefore, they persist and any systemic contact with this common antigen might cause a recurrence of ocular inflammation purely on an allergic basis. Recent work in rabbits and guinea pigs has demonstrated that this does, in fact, occur.[12] Rabbits injected with dead organisms intracutaneously and intraperitoneally as long as one year after the intravitreal inoculation of Toxoplasmas will develop a severe uveitis. This communication reports an attempt to determine the effect of corticosteroids on some of these hypersensitivity factors of toxoplasmosis uveitis.

In addition, although hypersensitivity appears to be important in toxoplasmosis, the disastrous effects of corticosteroids on some acute infections of the eye have been demonstrated.[13] Since steroid hormones might benefit the hypersensitivity component of recurrent toxoplasmosis uveitis, and since it is impossible at present to determine the relative importance of proliferating organisms as opposed to allergy, it appeared important to ascertain (1) whether in the face of proliferating organisms corticosteroids made the local disease better or worse; (2) whether they facilitated spread of the disease; and (3) whether with this study of corticosteroids further insight might be gained as to the role of hypersensitivity in contributing to the destruction seen in acute as well as chronic toxoplasmosis.

* From the Uveitis Laboratory of the Howe Laboratory of Ophthalmology of the Massachusetts Eye and Ear Infirmary. This work was supported by U. S. Public Health Service Grant B 2036 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health.

Methods and Materials

To investigate the effect of corticosteroids on acute ocular toxoplasmosis, three groups of albino rabbits weighing between one and two kilograms were studied. The first two groups were all inoculated intravitreally in both eyes with .05 cc. of a suspension of mouse brain containing Beverly strain Toxoplasmas. This strain generally infects and forms cysts, but does not kill rabbits. On these 19 infected rabbits a blind study was done; 11 were selected at random by a technician for treatment with hydrocortisone. Five were treated with 50 mg. of hydrocortisone injected subcutaneously twice a day, and six were similarly treated with 25 mg. once a day. All rabbits were treated from the day of infection for 12 days, being observed and examined biomicroscopically daily. The observer had no knowledge of which animals were in the treatment group and which were untreated but infected controls.

A third group of 12 rabbits from the same shipment were not infected but were treated with hydrocortisone in a like manner for 12 days—six with 50 mg. twice a day and six with 25 mg. once a day—to determine whether corticosteroids alone might be lethal to rabbits.

A fourth group of four rabbits were infected, as described previously, seven months before the experiment and then treated with 25 mg. of hydrocortisone a day to determine whether a chronic infection would be reactivated locally or systemically during or following steroid treatment. Two of the rabbits were subsequently killed and the presence of infection was verified by mouse inoculation of the ground-up eyes and brains. The mice died 12 days after infection, suggesting that residual infection was appreciable.

Results

The clinical evaluation of steroid treated and control rabbits conducted on a blind basis revealed no significant gross or biomicroscopic difference between the control and the treated infected groups. In all rabbits, however, the vitreous soon became opaque, the retina was not visible by direct or indirect ophthalmoscopy and clinical evaluation was incomplete and unsatisfactory.

Histopathologic examination of several sections from 20 eyes was also done without knowledge of whether the rabbits under study had been treated. As might be expected, retinal and uveal pathology was seen in both groups, with discharge of inflammatory cells into the vitreous and generally with protein and cells in the anterior chamber. The striking finding, however, was that in the 11 rabbits of the cortisone treated group, retinal lesions, although sometimes large, were generally localized, leaving areas of normal retina and uvea (Table 1). In the nontreated enucleated group, however, at comparable times after inoculation, retinal destruction and infiltration were at first perivascular but later became more generalized and gliosis of the nerve fiber layer was much more diffuse, as was uveal infiltration (fig. 1-4). Especially in those rabbits killed 14 days after inoculation, the disorganization seen in the nontreated group was considerably more massive than that observed in the more focal lesions of the rabbits receiving steroids. In the nonsteroid group the extensive lymphocytic and plasma cell infiltration of the retina and uvea and the general disorganization of the retina were more remarkable.

Although corticosteriod therapy appears to preserve the uvea and retina, the general health of the animals fared less well. All of the treated animals died within 14 days of infection, whereas no untreated infected ani-

TABLE 1

HISTOLOGIC EXAMINATION OF RABBITS WITH OCULAR TOXOPLASMOSIS

	Number of Eyes With Diffuse Uveal and Retinal Infiltration and Disorganization	Number of Eyes With Focal Retinal Lesions
Untreated	9	0
Treated with hydrocortisone	1	10

Fig. 1 (Kaufman). In many animals with acute ocular toxoplasmosis that have not received corticosteroids there is marked round-cell infiltration of the retina, choroid and vitreous and striking proliferation and gliosis of the superficial retina.

mals died and only one of the uninfected steroid treated group died (Table 2). In the brains and retinas of several of the infected steroid animals which were observed microscopically, many proliferative Toxoplasmas were seen.

TABLE 2

THE EFFECT OF CORTICOSTEROIDS ON OCULAR TOXOPLASMOSIS

Dose of Hydrocortisone	Acutely Infected Rabbits		
	Alive	Dead	% Dead
50 mg. twice a day	0	5	100
25 mg. once a day	0	6	100
None	12	0	0
Dose of Hydrocortisone	**Uninfected Rabbits**		
	Alive	Dead	% Dead
50 mg. twice a day	5	1	16.7
25 mg. once a day	6	0	0
Dose of Hydrocortisone	**Chronically Infected Rabbits**		
	Alive	Dead	% Dead
25 mg. once a day	4	0	0

Despite the fact that corticosteroid treated rabbits with ocular toxoplasmosis died, humans with presumed ocular toxoplasmosis who are treated with corticosteroids generally survive.[14] It seemed likely that this was not simply a species difference but rather might be related to chronicity of infection (human ocular toxoplasmosis appears to be a manifestation of chronic rather than acute infection) with the immune protection this implies. In addition, the number of proliferative organisms might be decreased in chronic infection. To study this, four rabbits with chronic toxoplasmosis of both eyes were treated with hydrocortisone. None of them died and none developed a recurrence or worsening of the ocular disease during or after corticosteroid therapy, although the presence of residual infection was proven by isolation of the organism from eyes and brain. This is in contrast to the experience of

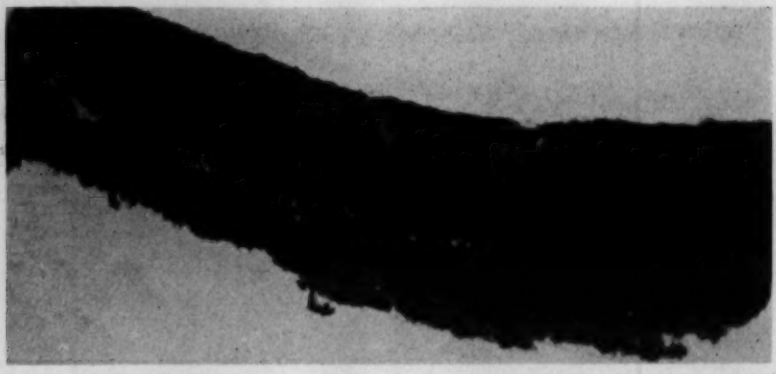

Fig. 2 (Kaufman). Without hydrocortisone areas of retina and choroid sometimes become so infiltrated and disorganized that they appear to fuse.

Frenkel[8] who found that some hamsters with generalized systemic toxoplasmosis even in the subacute or chronic phase became worse and died after corticosteroid therapy.

The severe recurrence of allergic uveitis produced by challenging with dead Toxoplasmas rabbits that have inactive chronic ocular toxoplasmosis has been described.[12] The administration of 25 mg. of hydrocortisone to two rabbits subcutaneously twice a day beginning two days before the antigenic challenge appeared to lessen, but did not prevent, the uveitis.

Discussion

There can be little doubt that corticosteroids decrease host resistance to acute infection by proliferating toxoplasmas and that chronically infected animals appear to be resistant to these adverse steroid effects. The

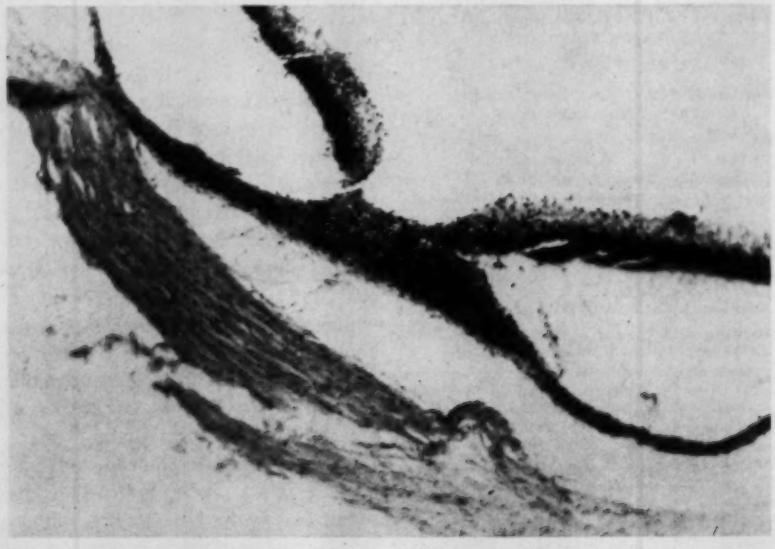

Fig. 3 (Kaufman). In rabbits treated with hydrocortisone large areas of uvea and retina appear normal or nearly normal, and lesions are more focal.

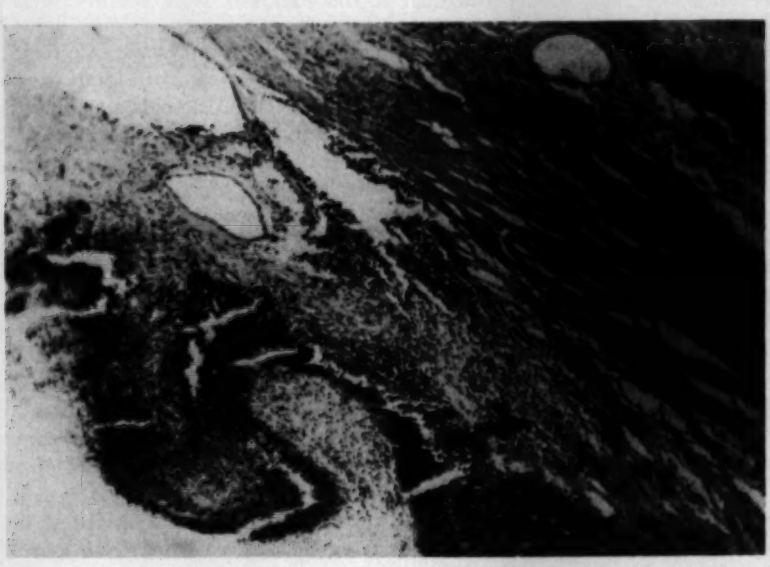

Fig. 4 (Kaufman). Without hydrocortisone the ciliary body and iris are commonly infiltrated with lymphocytes and plasma cells.

two most significant differences between these chronically infected rabbits and the acutely infected rabbits appears to be the presence of immunity and the possibility that fewer proliferating organisms were present to spread the disease.

Clinical experience with human toxoplasmosis indicates that the uveitis observed in most cases of adult ocular toxoplasmosis occurs as a manifestation of the chronic phase of the disease. Frenkel was the first to correlate a high incidence of skin sensitivity to toxoplasmin and the occurrence of uveitis,[15] and subsequently, the studies of Woods[16] and later Kaufman[17] show that the vast majority (up to 95 percent) of patients with uveitis and positive Toxoplasma dye tests also have positive skin tests.[18] Considerable evidence indicates that the skin test does not become positive for many months after acute infection, so that a positive skin test alone implies chronic disease.[9]

If an analogy to human disease is possible, therefore, it would suggest that acute disseminated infection (such as might occur in congenital toxoplasmosis) in the absence of effective immunity is made lethal by corticosteroids, but that the ocular disease of chronic toxoplasmosis does not exacerbate or become lethal with steroid therapy. This seems consistent with clinical experience in humans.[14]

More interesting perhaps than the effect of corticosteroids on survival was the striking decrease in retinal destruction and retinal and uveal infiltration following therapy. Although the infecting suspension was impure and the reaction might in part be due to trauma and tissue necrosis, it seems likely that to some extent the injection of toxoplasmas into the vitreous causes a hypersensitivity reaction similar to the injection of any antigen into the vitreous. The primary cellular destruction of a virulent organism is combined with this hypersensitivity reaction that compounds the pathologic changes.

Silverstein, Zimmerman[10,11] and others have described the pathologic alterations observed when pure antigens are injected into the vitreous. After injection, a reaction characterized by infiltration and later, granuloma formation and gliosis can be observed. Al-

though in our injected rabbits both the mouse brain and the organisms might serve as antigens, it seems likely that any organism invading the eye might take part in such a reaction, presumably as early as five to seven days after the initial invasion and that such responses might be important in the ocular destruction and loss of vision. In this respect the eye appears similar to the infant brain, in that the stenosis of aqueduct of Sylvius which, in cases of congenital toxoplasmosis, leads to hydrocephalus is a proliferative and gliotic response occurring in the absence of much local necrosis. This gliotic reaction is felt to be caused by hypersensitivity.[8] The prevention of ocular damage, such as this, with corticosteroids suggests that the destruction of ocular tissue consequent to infection of the eye by many types of organisms may be, in a large part, the result of hypersensitivity and raises the question as to whether the addition of corticosteroids to specific therapy might be beneficial not only in the case of chronic and recurrent infection, but even in relatively acute infections.

Summary

Allergic phenomena appear to play a major part in causing recurrences of ocular toxoplasmosis and in contributing to the destruction seen in chronic infection. A study was undertaken, therefore, to determine whether corticosteroids might be beneficial or detrimental to acute ocular infection or might cause an exacerbation of chronic ocular toxoplasmosis.

Hydrocortisone proved lethal to rabbits acutely infected with Beverly strain Toxoplasmas whereas the controls survived. In the steroid-treated rabbits, however, retinal and uveal infiltration and retinal destruction were more focal and less severe. It is suggested that the more extensive destruction in the untreated eyes may be, at least in part, on an allergic basis, similar in many ways to that observed after injection of nonliving antigens into the vitreous. If specific therapy is available to prevent spread of the organism, corticosteroids may be a useful adjunct in minimizing ocular damage.

Since human ocular toxoplasmosis appears to occur in the chronic phase of the disease, chronically infected rabbits were treated with hydrocortisone. Neither systemic illness nor recurrence of ocular inflammation was observed. Allergic recurrences of chronic ocular toxoplasmosis were not completely prevented by 25 mg. of hydrocortisone given subcutaneously twice a day.

243 Charles Street (14).

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Discussion

Dr. Ralph W. Ryan (Morgantown, West Virginia): In discussion of this fine research paper, I have three causes for gratification: (1) the pleasure of being asked to comment on a subject in which I have long been interested, (2) the fact that our knowledge of the interaction between host, organism and hormone in toxoplasmosis has been advanced, (3) The anticipation that our treatment of human ocular toxoplasmosis will become more effective because of such research as this.

The most interesting finding of this experiment was the sparing of retinal tissue and localization of lesions by corticosteroid therapy. This is contrasted with diffuse destruction of retinal tissue in the untreated group. This finding makes us more able to distinguish between the relative damage from actual tissue invasion by organisms and the more generalized destruction due to host inflammatory or allergic response.

This insight is of special interest to ophthalmology where the host's protective mechanisms against disease may destroy vision as effectively as the direct effects of the disease.

The use of corticosteroid therapy affects primarily host immunity. The inhibition of mobilization of polymorphonuclear leucocytes may thus protect the retina against disorganization from massive infiltration by these cells. Similarly, lymphocytic and plasma cell infiltration is discouraged.

It has been shown that the fixed macrophages are also inhibited by corticosteroids and that antibody production is inhibited. It would, therefore, appear that the host animal is deprived of most of its protection against direct invasion of organisms. This would explain the lethal effect on all the corticosteroid-treated animals with acute infection.

It would have been interesting if studies had been possible on the relative parisitemia in the blood of treated and untreated animals during the 12-day period. Since the reticuloendothelial system could be expected to have been inhibited, parisitemia should have been increased by the corticosteroid therapy. Similarly, local proliferation of organisms would be expected to have been greater in the treated animals. An assay of numbers of toxoplasma organisms in the retinal lesions of the treated and untreated animals would have been of interest.

It seems that the effect of corticosteroids in acute toxoplasmosis temporarily benefits the eye by preventing the inflammatory response which would quickly destroy its function. Since invasiveness of the disease is not inhibited, but rather the host's defenses against invasion are inhibited, the ultimate result of corticosteroid therapy in the acute disease is unsatisfactory. This, of course, is the finding in studies of other infectious diseases.

Of greater import to clinicians is the effect of corticosteroids on chronic and recurrent toxoplasmosis. Dr. Frenkel's studies on hamsters would suggest that the disease might be exacerbated eventually by use of heavy dosage of corticosteroids alone.

In Dr. Kaufman's experiment on four rabbits infected seven months previously, it is noteworthy that the two animals which were killed both gave positive mouse inoculations. If the corticosteroid therapy had been continued until the residual antibodies had been greatly reduced, one might wonder if a fulminating invasion of proliferative organisms would ensue.

From the clinician's viewpoint, we have learned that corticosteroids cause at least temporary reduction in retinal tissue damage from acute ocular infections with Toxoplasma in rabbits. We have little reason to doubt that humans react somewhat similarly.

Inhibition of the inflammatory response should serve a useful purpose in chronic or recurrent retinochoroiditis in humans if it can be carried out without endangering the host's defense against the organisms. Concurrent therapy with pyrimethamime and sulfonamides is the present logical solution to this problem. Such combination therapy with pyrimethamine, sulfonamides and corticosteroids has been tried with promising results by a number of clinicians. However, results with any treatment in this disease are difficult to evaluate fully.

Both the findings of this and other similar experiments with corticosteroid therapy and the ex-

perience to date with clinical use of corticosteroids suggest the possible danger of heavy long-continued therapy with corticosteroid drugs alone in the presence of infectious disease without controlling proliferation of organisms by other means.

DR. DAN M. GORDON (New York): This has been a beautiful presentation of a beautifully done piece of work. I would like to correct the speaker and Dr. Ryan on one point. They state that steroid treated ocular toxoplasmosis patients "generally" survive. They always survive.

It has been my clinical impression that Pyrimethamine and sulfa do not benefit these patients or prevent recurrences to any appreciable extent. Since the disease in adults is practically always allergic in nature, one should not expect antimicrobials to be of value. I feel that most uveitis is allergic in nature.

DR. HERBERT E. KAUFMAN (closing): I would like to thank Dr. Ryan for his very perceptive discussion.

Although we did no studies on parasitemia, it was certainly my impression in observing both sections of eye and brain that there were far more proliferating parasites in the treated than in the untreated animals, and I think the conclusion is probably justified that the organisms did multiply much more widely.

We have no data on the possibility of an exacerbation of chronic toxoplasmosis after long-term therapy. There is no question, however, that acutely infected and chronically infected animals are different in their response to corticosteroid therapy. I think host resistance must be, at least in part, responsible for this difference.

Dr. Ryan suggested the use of both pyrimethamine and sulfonamides as well as corticosteroid therapy in the treatment of clinical toxoplasmosis. Although our knowledge at present is incomplete, I think that this is a logical method of treatment and use such a regimen myself.

I agree with Dr. Gordon as to the usefulness of corticosteroids and the importance of allergy. My clinical impression about the usefulness of specific therapy is different from his, however, and I know of no objective evidence to support his conclusion. As stated I use a combined therapy.

CORNEAL DESTRUCTION BY EXTRACTS OF CEPHALOSPORIUM MYCELIUM*

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INTRODUCTION

There has been an apparent increase in the incidence of fungal keratitis since steroid preparations have been added to the ophthalmologist's armamentarium. Recently reported clinical experiences with this disease have been more numerous.[1-5] Experimental[6-8] and statistical[9] observations have supported the contention that these therapeutic agents, if used indiscriminately, can play a significant role in the establishment and/or enhancement of fungal keratitis. This apparent rising incidence combined with the marked severity of the disease has resulted in a potentially common and serious problem for the ophthalmologist.

The purpose of this study, therefore, has been to investigate the basic mechanism whereby fungi are able to destroy corneal tissue. Since an enzymatic protein, a protease, has been shown to be responsible for the corneal destruction observed in Pseudomonas keratitis,[10] it was postulated that such an enzymatic factor may be present in Cephalosporium keratitis. An understanding of the pathologic events at the cellular and ultimately at the biochemical level, in this form of keratitis, may be valuable in the development of adequate therapeutic and preventive methods.

MATERIALS AND METHODS

A species of Cephalosporium isolated from a clinical case of keratitis was selected as the test organism in this investigation. Bulk quantities of fungus were grown for seven days at room temperature in 100 ml. volumes of a modified liquid Sabouraud's medium in Roux bottles. The composition of the growth medium is as follows:

Dextrose—40 gm./liter.

* From the Department of Ophthalmology, Tulane University School of Medicine. This investigation was supported in part by research grant B-1307 (C2) and B1823 from the National Institutes of Health, Public Health Service, and Fight for Sight Fellowship SF-167 from the National Council to Combat Blindness.

Difco Dehydrated Nutrient Broth—10 gm./liter pH adjusted to pH 4-6.

The cultures were incubated at room temperature for one week. This gave optimum yield of mycelium with the least amount of sporulation. Before harvesting the mycelium, the cultures were examined to eliminate the possibility of bacterial contamination. The mycelium was harvested by filtration and stored at −20°C.

Mycelial extracts were prepared by mechanical disruption of the mycelium, using an abrasive and grinding the frozen material in a chilled mortar. Two parts of abrasive, either Alcoa Alumina A301 or powdered glass were used per one part of mycelium. The most convenient weight of material to work with was found to be 80 to 90 gm.

The mixture was immediately extracted with approximately 50 ml. of cold normal saline and centrifuged at 7000 × g. at 0°C. for 30 minutes. This yielded a greenish to yellow opalescent supernatent with a very small amount of flotation material. The supernatent extract was dialyzed against cold distilled water for 48 hours.

The activity of each crude extract and all subsequent fractions was tested in vivo by intracorneal inoculation of 0.05 ml. volumes into the eyes of healthy mature anesthetized 3.5 kg. rabbits. Extracts which did not possess ulcerative activity were discarded.

The initial crude extracts were lyophilized in order to obtain maximum concentration of cellular protein. However, it was found that sufficient ulcerative activity could be demonstrated in the unconcentrated extracts. Later extracts were not concentrated.

The active principle was fractionated using ammonium sulfate precipitation methods. This was accomplished by saturating the mycelial extract with varying concentrations of ammonium sulfate and collecting the material which precipitated at each of the saturation levels. Levels of 30, 60 and 80 percent saturation, with respect to ammonium sulfate, were empirically chosen. Precipitation was allowed to proceed for 12 to 24 hours in cold temperature. The precipitates were collected by centrifugation at 12,000 × g. for 30 minutes and dissolved in 2.0 ml. of cold saline at pH 7.0. Activity of each fraction was determined by intracorneal inoculation of rabbit eyes. All extracts and fractions were checked for bacterial contamination by plating on appropriate media. Eyes which were ulcerated were likewise cultured to eliminate bacterial contamination.

The relatively large quantity of $(NH_4)_2SO_4$ in the various fractions was found to have no adverse effect on corneal tissue; however, it did interfere with the various enzymatic assays. This difficulty was overcome by dialyzing active fractions against a phosphate buffer system, pH 7.0 under refrigeration for 24 hours. This procedure not only removed the interfering agent but also helped maintain stability of the protein solution.

The active fraction was assayed for the following enzymatic systems: (1) acid and alkaline phosphatase; (2) deaminase; (3) gelatinase; (4) mucopolysaccharidase; and (5) proteinase.

Proteolytic substrates consisted of rabbit and chicken corneal proteins, gelatin and casein. The soluble and insoluble rabbit and chicken corneal protein fractions were prepared according to the methods of Guidry[11] and Fisher.[12] A 5.0 percent gelatin prepared in distilled water solution was used. Casein was diazotized according to the methods of Tomerali[13] and Fisher.[10] Mucopolysaccharide substrate consisted of hyaluronic acid and heparin.

Hyaluronic acid was prepared from beef vitreous humor. The heparin substrate was a commercial preparation, Abbott, 5,000 units U.S.P. per ml.

Results

Crude extract studies

Intracorneal inoculation of 0.05 ml. of crude saline mycelial extract into rabbit eyes produced corneal destruction causing an ul-

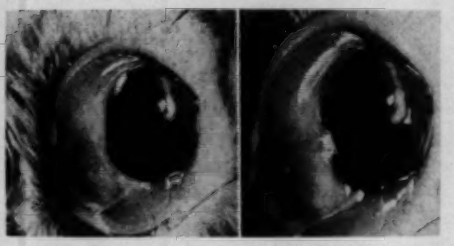

Fig. 1 Fig. 2

Figs. 1 and 2 (Burda and Fisher). Corneal destruction four hours after intracorneal inoculation of 0.05 ml. Fraction II.

ceration, exudative liquefaction and some opacification. The ulceration was not as striking as the exudative liquefaction. This appeared as a "stringy" mucoid material which oozed from the injection site. (Same as shown in fig. 1 and 2). Such damage was not noted when the culture supernate was intracorneally injected, indicating the active material apparently is not elaborated into the growth medium.

Maximal corneal destruction was observed in two to four hours. This process appeared to be a self-limited one, in that only corneal scarring resulted. Complete destruction or damage to the anterior chamber of the eye was never noted.

The degree of corneal damage varied from extract to extract. Quantitative determination of the potency of the extracts was not made, therefore no precise correlation could be made between the extent of damage and potency. However, random protein concentration determinations on the crude extracts ranged between 0.66 and 1.08 mg./ml., giving some idea of the protein concentration of the crude material at which damage was initiated.

Fractionation studies

Fractional precipitation of the active crude extracts with various concentrations of $(NH_4)_2SO_4$ yielded three precipitates corresponding to 30, 60 and 80 percent $(NH_4)_2SO_4$ saturation. Heaviest precipitates were usually obtained at 30 and 60 percent saturation while very little material precipitated at the 80 percent level. The saline solution of these precipitates were designated as Fraction I, II and III respectively. Fraction IV was the supernate of 80 percent precipitation. A small amount of dark flotation material was occasionally obtained on the supernate after centrifugation of the 30 percent precipitation. This apparently was largely lipid material and was discarded.

The activity of each of these fractions was determined by intracorneal inoculation. As shown in Table 1 and Figures 1 and 2, the fraction obtained at 60 percent saturation was the only one found to be active in corneal destruction. The destruction caused by this fraction was similar to that observed in the crude preparation, that is: exudative liquifaction, ulceration and some corneal opacification. Again the degree of damage varied from extract to extract and potency was not quantitated.

Enzymatic studies

Further attempts at identification and characterization of the active factor contained in Fraction II were made. This problem was approached by studying various enzymatic activities of the fraction. The following rather broad spectrum of determinations was selected initially in the hope that the responsible factor would fall into one of these enzymatic categories.

1. Phosphatase activity

Colorimetric determination of acid and alkaline phosphatase activity[14] was carried out on 0.4 ml. of 1 : 1,000 dilution of Fraction II. The release of p-nitrophenol using disodium p-nitrophenyl phosphate as the substrate was followed. A significant amount of acid phosphatase was shown to be present (fig. 3); however, no alkaline phosphatase was detectable.

2. Deaminase and gelatinase activities

Deaminase activity was determined by microdiffusion analysis (Conway).[14] Gelati-

TABLE 1

INTRACORNEAL INOCULATION OF CEPHALOSPORIUM MYCELIUM CRUDE EXTRACT AND ITS FRACTIONS

Fraction	$(NH_4)_2SO_4$ gm./1000 ml.	Percent $(NH_4)_2SO_4$ Saturation	Corneal Destruction			Culture
			Ulceration	Liquefaction	Opacification	
Crude extract	0	0	+	+	+	−
I	176	30	−	−	−	−
II	198	60	+	+	+	−
III	143	80	−	−	−	−
IV	143	80	−	−	−	−

nase determination[14] was done by conventional viscometric methods employing Ostwald viscometers. No significant quantity of gelatinase or deaminase was demonstrable by these methods.

3. Mucopolysaccharidase activity

The mucopolysaccharidase activity of the active fraction was determined by application of Parker and Johnson's method[15] of submicro glucose determination. Beef vitreous hyaluronic acid, heparin and rabbit corneal proteins served as substrates. No significant increase in number of reducing groups in the form of N-acetylglucosamine was detectable.

Viscometric determination[16] of mucopolysaccharidase activity using beef vitreous hyaluronic acid as the substrate was also nonrevealing.

The inherent difficulty in assaying for this enzyme is realized and these assays by no means exclude the presence of the enzyme.

4. Proteolytic activity

Two proteolytic assay methods were employed. These were the colorimetric measurement of the release of ninhydrin positive materials and the colorimetric determination of digested diazotized protein (diazotized casein).[10]

The incubation mixture for the ninhydrin assay consisted of 1.5 ml. substrate, 1.5 ml. Tris* buffer 0.1 M pH 7.0 and 1.0 ml. of Fraction II solution. The buffer and substrate were incubated for 15 minutes at 37°C. after which time Fraction II ("enzyme") was added. Samples, 0.1 ml. of the reagent mixture, were removed at indicated time intervals, added to 0.1 ml. of ninhydrin reagent and placed in a boiling water bath for 20 minutes. After cooling, 5.0 ml. of an equal parts mixture of n-propanol and distilled water was added as the dilutent. Optical density was read at 570 mμ wave-length in the Coleman Universal Spectrophotometer.

Soluble as well as insoluble rabbit corneal proteins were actively degraded as shown in Figure 4. Strong proteolytic activity was also demonstrated against water soluble chicken corneal proteins and 5.0 percent gelatin solution (fig. 5). Assays on heated Fraction II solutions served as controls where no proteolytic activity was detectable.

The reaction mixture for the diazocasein degradation assay was composed of 5.5 ml. of substrate (diazocasein 10 mg. per ml.), 5.5 ml. of Tris buffer 0.1 M pH 7.0, 4.4 ml. of distilled H_2O and 1.0 ml. of Fraction II.

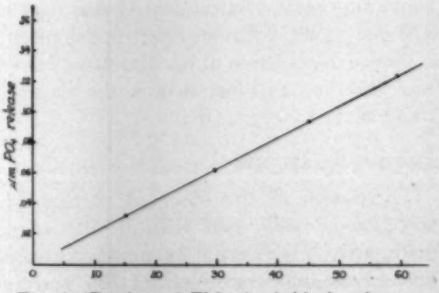

Fig. 3 (Burda and Fisher). Acid phosphatase activity of 1:1100 dilution of Fraction II.

* Tris (hydroxyethyl) aminomethane.

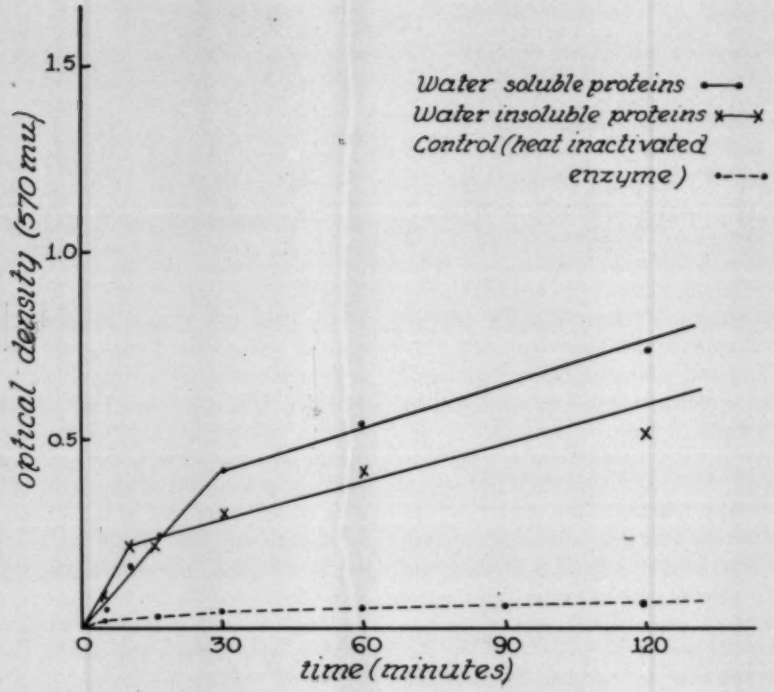

Fig. 4 (Burda and Fisher). Proteolytic activity of Fraction II against rabbit corneal proteins (ninhydrin assay).

Substrate and buffer were incubated for 15 minutes at 37°C. Fraction II ("enzyme") was then added and at indicated time intervals 1.5 ml. of aliquotes of the mixture were removed and placed into 3.5 ml. of 5.0 percent trichloroacetic acid. The precipitated undigested substrate was removed by centrifugation. The color was developed by adding 2.5 ml. of 0.5 N NaOH to 2.5 ml. of the above supernate. Optical density was read at 420 mμ. in the Coleman Spectrophotometer.

Active degradation of the diazotized casein was observed as illustrated in the time-activity curve of Figure 6.

Inhibition studies

Correlation of the observed corneal destruction in rabbit eyes with in vitro enzymatic activity was made by employing various enzyme inhibitors. NaF 0.1 M., Versene 0.1 M pH 7.2, p-chloromercuribenzoate 0.0026 M (0.1 percent), $HgCl_2$ 0.01 M and heat were the inhibitors selected for these experiments.

Attempted inhibition of the acid phosphatase activity involved incubating 0.1 ml. of NaF with 0.1 ml. of Fraction II for 20 minutes at room temperature. Intracorneal inoculation of 0.05 ml. of this mixture into rabbit eyes resulted in corneal destruction; however, in vitro phosphatase activity was inhibited.

Versene, p-chloromercuribenzoate, $Hg\ Cl_2$ and heat were employed as proteolytic inhibitors. In vitro inhibition of diazocasein degradation was determined using essentially the same procedure as that employed in the proteolytic assays. Smaller volumes of reagents were used and only one determination was carried out on the reaction mixture, that is: at the end of one hour. In this instance the incubation mixture consisted of

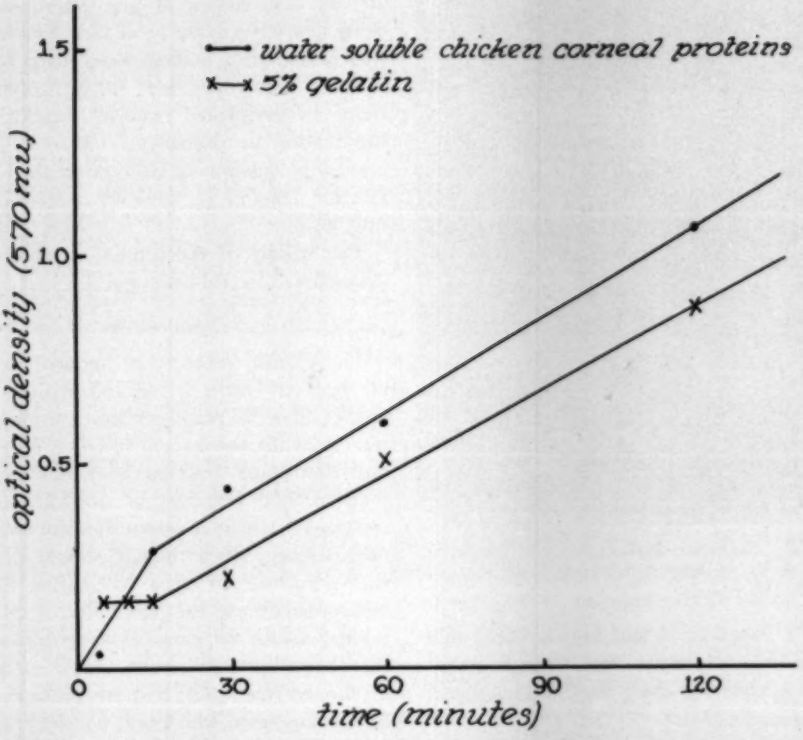

Fig. 5 (Burda and Fisher). Proteolytic activity against water soluble chicken corneal proteins and 5.0 percent gelatin.

0.5 ml. diazocasein (20 mg./ml.) 0.5 ml. Tris buffer 0.1 M pH 7.0, enzyme 0.1 ml. and distilled water and/or inhibitor to make a total volume of 1.5 ml. The respective volumes and concentrations of each inhibitor is given in Table 2.

No significant inhibition of in vitro proteolytic activity was obtained using Versene or p-chloromercuribenzoate. However 0.03 ml. Hg Cl_2* 0.01 M (final dilution of 1 : 50) was shown to significantly inhibit the digestion of diazocasein by Fraction II. Approximately 34.2 percent inhibition was detected. Correlation of this inhibition with the inhibition of corneal destruction was made by intracorneal injection of 0.05 ml. of a mix-

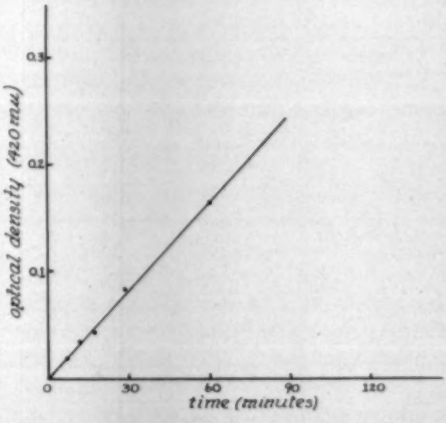

Fig. 6 (Burda and Fisher). Degradation of diazocasein by Fraction II.

* A 1:50 dilution of a 0.01M $HgCl_2$ solution was observed tc have no deleterious effect on the cornea.

Fig. 7 (Burda and Fisher). Corneal destruction inhibited by treating Fraction II with mercuric chloride (four hours).

ture of Fraction II and $HgCl_2$ (final dilution 1 : 50). No significant corneal destruction was observed other than minimal opacifications (fig. 7).

The effect of heat on in vitro proteolytic activity of Fraction II was determined by using ninhydrin assay procedure. Fraction II was heated in a boiling water bath for 15 minutes. Ninhydrin assay for proteolytic activity on the heated material demonstrated inactivation of proteolysis (fig. 4). Intracorneal inoculation of 0.05 ml. of the heated fraction resulted in minimal corneal injury (fig. 8).

The results of the inhibition studies are summarized in Tables 2 and 3.

Discussion

The corneal destruction initiated in rabbit eyes by intracorneal inoculation of a crude saline Cephalosporium mycelial extract, and the subsequent isolation of a corneal destroying fraction from this extract, indicates the presence of an intracellular component which is responsible for the corneal damage. Even though corneal damage was not observed upon inoculation of the culture supernate, the possibility of an exocellular factor or enzyme being elaborated into the medium still exists. Small quantities of the enzyme would require concentration of the supernate which was not done in this study. Also, as emphasized by Waksman,[17]

TABLE 2

Summary of results from in vitro inhibition experiments

Inhibitor			Enzyme Prep. (ml.)	Substrate	H_2O (ml.)	Optical Density
Chemical	Conc. (molar)	Volume (ml.)				
Sodium fluoride	0.1	0.1	0.1	*p*-nitrophenyl phosphate	—	0
		0.0	0.1		—	140
Versene	0.1	0.1	0.1	Diazocasein	0.4	0.16
		0.0	0.1		0.5	0.16
p-chloromercuribenzoate	0.0026	0.4	0.1	Diazocasein	0.0	0.31
		0.0	0.1		0.4	0.36
Mercuric chloride	0.01	0.03	0.1	Diazocasein	0.37	0.125
		0.0	0.1		0.4	0.190
Heat	100°C. for 15′		0.5	H_2O soluble rabbit corneal proteins	0.7	0.06
	Unheated		0.5		0.7	0.53

an enzyme which functions as an intracellular enzyme at one stage of the growth process may be liberated into the medium as an extracellular enzyme at a later stage of the growth. Therefore the mycelium should be studied for presence of the factor in the early growth stages as well as the medium in later growth stages. It is interesting to note that in our studies, extracts prepared from mycelium older than 10 days possessed very little corneal destroying properties, indicating the possibility that at this stage of growth the enzyme becomes extracellular. Studies on the relationship of the extracellular to intracellular ratio of enzyme concentration to the type of medium, and corneal destruction would be desirable.

The demonstration of a large quantity of acid phosphatase in the cellular components of the fungus is not unusual, since phosphatase plays an active and essential role in cellular metabolism. It was postulated that this phosphatase may be the active factor in causing corneal destruction through some type of energy-releasing mechanism. However, inhibition of its activity by a suitable inhibitor did not prevent corneal damage, indicating another enzyme must be responsible for corneal damage.

The biochemical nature of corneal ground substance has been identified as a mucopolysaccharide consisting of monosulfuric esters of hyaluronic acid.[18] The demonstration of depolymerization and breakdown of this hyaluronosulfuric acid complex by pneumococcal and testicular hyaluronidase by these investigators prompted investigation of the Cephalosporium extracts for the presence of a mucopolysaccharidase. However, no significant quantity of this enzyme was detected by our methods, using beef vitreous hyaluronic acid and rabbit corneal crude extracts as substrates.

TABLE 3

RESULTS OF INTRACORNEAL INOCULATION OF ENZYME-INHIBITOR COMBINATION

Enzyme Plus Inhibitor	Corneal Damage (in situ)
Sodium fluoride	+
Versene	+
P-chloromercuribenzoate	+
Mercuric chloride	−
Heat	−

Fig. 8 (Burda and Fisher). Intracorneal inoculation of 0.05 ml. of heat activated Fraction II (four hours).

Woodin[19, 20] emphasizes reconsideration of Meyer's demonstration of hyaluronidase activity against corneal polysaccharides. Hyaluronidase preparations from various sources failed to demonstrate this effect against in situ corneal polysaccharides. Better purified hyaluronidase preparations, hyaluronic acid substrates and improved assay methods may provide the needed information concerning the exact role of this enzyme system in various corneal inflammations.

Proteolytic studies of the mycelial extracts proved to be the most rewarding. The active degradation of rabbit and chicken corneal protein, gelatin and casein by Fraction II indicates the presence of a proteolytic enzyme. However, since these substrates are proteins and presently not well defined chemically, accurate classification of this enzyme on the basis of substrate specificity is not possible. With the aid of a more purified enzyme preparation, it is hoped that

kinetic and comparison studies with known enzymes will facilitate better enzymatic classification. The question of whether this is a constitutive or adaptive enzyme remains to be investigated. Present evidence would place the enzyme in the general class of constitutive proteinases.

The presence of proteinases and peptidases in fungi has been established. Johnson and Peterson[21] studied the Aspergillus parasiticus proteolytic system by using aqueous extracts of the mold which were purified by acetone precipitation and ultrafilteration. A proteinase was identified along with peptidases possessing properties similar to that of the aminopolypetidase, the carboxypolypeptidase, and the dipeptidase of the animal digestive system. An investigation of the proteolytic system of 30 common molds by Berger et al.[22] also revealed the presence of a proteinase plus two additional enzymes which hydrolyze di- and triglycine peptides. Studies are presently being conducted to determine if there are such peptidases in Cephalosporium and if so their relationship to corneal destruction will be investigated.

The inhibition studies provided good correlation between proteolytic activity and corneal destruction. This was evidenced by the in vitro inhibition of proteinase activity by $HgCl_2$ and heat and the subsequent demonstration that enzyme preparations treated with these inhibitors failed to destroy rabbit corneal tissue. Since chelating agents did not inhibit proteolysis, a metal prosthetic group or cofactor may not be necessary for the enzyme's activity. Inhibition of enzyme action by heat inactivation is nonspecific. It merely denatures any protein moiety and should not be regarded as truly specific for a proteinase.

The mechanism whereby normal corneal integrity is disrupted in clinical fungal keratitis may reside in the proteolysis of either the lamellar collagenous bundles or the glycoproteins of the mucoid ground substance by this proteinase. Both soluble and insoluble corneal proteins were shown to be actively degraded by the proteinase. The insoluble proteins are probably largely collagen[12] and since 80 to 90 percent of the substantia propria is composed of this water-insoluble protein, it may be that the major site of action of this enzyme may be on this all-important stromal element. The activity of this enzyme against purified collagen preparations remains to be investigated. The water-soluble proteins are largely simple proteins and polypeptides and their hydrolysis is not an unexpected finding.

Clinical fungal keratitis is presumably the result of implantation of spores into a traumatized cornea. The proteinase may be elaborated during the initial phases of germination and proliferation of the spores, as studies of experimental fungal keratitis would seem to indicate.[8]

Invasion of the corneal stroma by the comparatively large mycelial filaments undoubtedly causes some mechanical damage. However, this factor alone could not account for the rapid onset of corneal destruction as seen in our experimental infections. The importance of this factor in corneal destruction will have to be evaluated also.

It is thought that if a mucopolysaccharidase were present, it might work synergistically with the proteinase in causing corneal damage. It is possible for the proteinase alone to affect the glycoproteins (mucopolysaccharide-protein complexes) of the ground substance. The hydrolysis of the protein portion of this complex may cause dissolution or a reduction in viscosity of the ground substance leading to corneal damage, manifested especially as exudative liquefaction. A study of the changes in corneal structure induced by this enzyme by various histochemical staining techniques may be of value in establishing the exact site of action.

Summary

Inquiry into the mechanism of the observed corneal destruction in Cephalosporium keratitis has been made. A saline mycelial extract was prepared by grinding

the mycelium with an abrasive. Intracorneal inoculation of this crude extract in rabbits produced corneal destruction in two to four hours, manifested as ulceration, some opacification and exudative liquefaction. Fractionation studies of the crude saline mycelial extract by precipitation with ammonium sulphate have yielded a fraction at 60 percent saturation with respect to ammonium sulphate, which has ulcerative activity.

Further attempts at identification and characterization of this active factor were made by studying the enzymatic activities of this fraction. A significant amount of acid phosphatase and proteolytic activity has been demonstrated in this fraction. No deaminase or mucopolysaccharidase activity was demonstrable. Gelatinase could not be detected by conventional viscometric determinations.

Strong proteolytic activity against various rabbit corneal proteins, chicken corneal proteins, gelatin and casein was demonstrated by the diazocasein degradation and ninhydrin assay procedures. Until further purification and kinetic studies can be made on this enzyme, it will have to be classed as a constitutive proteinase.

Correlation between the observed corneal destruction in rabbit eyes and this proteolytic activity was made by employing various enzyme inhibitors. The acid phosphatase activity was shown to be unrelated to in vivo corneal destruction.

The proteinase may be responsible for the pathologic picture of clinical Cephalosporium keratitis. The exact site action of this enzyme remains unestablished. Presumptive evidence would indicate the collagen bundles are affected; however, a disturbance in the mucopolysaccharide-protein gel state of the ground substance of the cornea cannot be definitely excluded.

Tulane University School of Medicine (12).

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Discussion

Dr. Wendell D. Gingrich (Galveston, Texas): I would like to congratulate the authors upon their investigational analysis of why certain fungi (this particular one) invade and destroy corneal tissue. These ordinarily nonpathogenic fungi under certain circumstances will actively invade and destroy corneal tissue.

My meager knowledge of biochemistry does not permit a critical analysis of their studies, but I think it is quite significant and it was demonstrated very nicely that the proteolytic enzyme was present within the mycelium, that this could be isolated in a distinct fraction, that the enzyme was active in vivo as well as in vitro, and by their inhibition studies and other studies the authors have shown that it is the proteolytic enzymes which are the destructive ones to corneal stroma.

I believe we were asked to present anything of a clinical nature, as we are meeting alternately with the clinical Section, and in this connection I have a case of Cephalosporium keratitis to present in a 72 year old white male. We first saw this corneal ulcer about three weeks after it began to progress, probably treated at least to some extent effectively in some way or other. The infection undoubtedly had progressed.

[Slide] The first slide shows the extent of the ulcer in this gentleman—somewhere around three to four weeks following its beginning.

[Slide] This shows some improvement following treatment with Amphotericin B, suspended in merthiolate 1:1000 administered simply in drops.

I do want to show you that there is increased luster of the cornea. There is apparent superficial healing in some measure, and this hypopyon is reduced and actually was membranous and could be pushed around somewhat.

This shows how this apparent improvement turned out to be not so great. The cornea became quite thin, and at the time we thought this was a descemetocele, but I will show you later that it was not actually a descemetocele but a dissolution of corneal stroma underneath epithelium which had healed over. This is after five days of that type of treatment. The treatment was then stopped, in the belief that we wanted to do nothing more to possibly harm the cornea. Sulfacetimide was therefore used hereafter with contraparesis.

[Slide] This shows how, three days later, this cornea perforated and the lens extruded. What you see is the vitreous face covered by imperfect corneal epithelium.

In retrospect, it is our belief that the organism was destroyed in the tissue, that the stroma simply dissolved by previous microscopic invasion not shown grossly by the mycelia of the fungus. This being the patient's only eye, it was not considered the end of it. We did not have a donor eye on hand at the time, but within 48 hours we performed a corneal transplant.

[Slide] For two days there was no change. The transplant was performed.

[Slide] This is the following day.

[Slide] This shows a culture of the organism.

[Slide] These are heads of spores or collections of spores. They are Cephalosporium. This shows you how this fungus can be identified as to species.

[Slide] This shows the distinct characteristics. These are hyphae which bear spores, but the spores are knocked off. Cephalosporium serrae are distinct from all other Cephalosporium species in that these particular conidiophores are branched. They bear conidiophores which here are knocked off, and you can see the branching of those filaments much better than when the heads are on.

[Slide] This shows a photograph taken by the referring physician approximately six months later, showing that the cornea has a measure of transparency and the man has some vision. This graft was grown into a hand-trimmed recipient cornea, and fortunately has at least this degree of clarity.

So, I believe I have demonstrated my first point, which is that these fungi invade microscopically to an extent that even though one kills the organism and for days there was no effective therapy, none following the transplant at all, the organisms may be killed but their effects allow devitalized cornea and perhaps the enzyme in addition to dissolve the stroma.

The last point I would like to show briefly on the next slide.

[Slide] You all know of the fine report of Banks-Anderson on the use of Amphotericin B in the cure of five corneal ulcers caused by various fungi. That is what led us to use Amphotericin B (Fungizone) against this Cephalosporium. This

all went so rapidly (it was ten days to perforation) that we did not get any studies on sensitivities performed in time for treatment.

This shows, however, that this Cephalosporium is totally insensitive to Amphotericin B in any possible therapeutic range. This is 1 mg per milliliter of medium which inhibited this small colony up here, probably being due to poor concentration of drug up there. The rest of these in the other tubes are not shown, but they grew the fungus quite profusely. This is sodium desoxycholate, a control.

Merthiolate, on the other hand, inhibited the growth down to a fraction of 1 microgram per milliliter.

[Slide] This shows Aspergillus in the same manner, which shows sensitivity to Amphotericin B as well as merthiolate.

This, I am sure, demonstrates that antibiotics are fine for what they will effect in adequate degree, but their coverage is incomplete, and there is a hole in any coverage by any antibiotic.

I wish once more to congratulate the authors on their fine investigation.

DR. C. D. BURDA (Closing): I wish to thank the discusser for a very fine correlation between our experimental studies and the cause of Cephalosporium keratitis. It was quite a nice case he presented.

The discussant admits the microscopic invasion of the corneal stroma by the fungus. I might add that in our experimental infections in rats under steroid therapy this is a pronounced feature of the keratitis. However we hope to determine whether this enzyme is actually elaborated in the cornea or whether this is a mechanical factor whereby the fungi cause perforation.

STUDIES ON EXPERIMENTAL OCULAR HYPERSENSITIVITY TO SIMPLE CHEMICALS*

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Washington, D.C.

The extensive investigations of Landsteiner and his coworkers[1] established a firm chemical basis for our understanding of the immunologic reactions into which simple, nonantigenic drugs and chemicals could enter. These investigations did much to clarify the problem of immunochemical specificity in terms of the molecular structures of these simple compounds. They further demonstrated that, while the simple chemical "hapten" alone is not antigenic, a conjugate of this hapten with protein would serve quite well as an antigenic stimulator of the immune mechanism.

With the rapid expansion of interest in hypersensitivity as a significant factor in the pathogenesis of disease, it was soon recognized that simple chemicals and drugs could contribute to allergic inflammatory reactions in two distinctly different fashions. The first of these is by the mechanism of anaphylactic or immediate hypersensitivity, mediated by circulating antibody. The interaction of antigen with antibody initiates a sequence of events which ultimately leads to such clinical manifestations as urticaria, atopic blepharoconjunctivitis, angioneurotic edema, hay fever, certain instances of inflammation of the uveal tract,[2] and so forth. With the recognition of tuberculin or "delayed" hypersensitivity as a phenomenon distinctly different from anaphylaxis, there developed an appreciation of a second, nonanaphylactic mechanism operating in the field of drug allergy. This is responsible for the contact dermatitis type of reaction, typified by poison ivy sensitivity, which is related to delayed microbiallergic hypersensitivity. Among the similarities between contact dermatitis and microbiallergic hypersensitivities are (a) the delayed onset of inflammation; (b) the lack of dependence of the response on circulating antibody (with the antibody-deficient agamma-globulinemic subject able to develop this type of immune response); and (c) the ability to transfer skin reactivity with lymphoid cells but not with serum from the sensitized donor.

Contact sensitivity to simple chemicals is

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† From the Immunochemistry and Ophthalmic Pathology Branches, Armed Forces Institute of Pathology, Washington, D.C.

characterized by certain peculiarities which set it apart from delayed responses to bacterial antigens. Firstly, by far the most favorable mode of induction is via the cutaneous route. Only exceptionally has this form of hypersensitivity been induced otherwise,[3] as for example with the use of adjuvant mixtures,[4] complex conjugates with erythrocyte stroma,[5] or collagen.[6] Secondly, and of more immediate interest, Eisen[7] has pointed out in a recent review that with respect to *elicitation* of the contact hypersensitivity response, "the skin appears to be unique." In no other tissue can this type of delayed sensitivity to simple chemicals be demonstrated experimentally. Thus, challenge of the hypersensitive subject with the contactant (the allergenic chemical which can induce sensitivity or elicit the contact response) by intravenous or intraperitoneal route, or even via the oral mucosa, is ineffective.

This latter point has led us to consider the possibility of eliciting a primary contact hypersensitivity response in ocular tissues. In line with current immunologic concepts, contact allergy of the eyelids should be easily produced experimentally, as it is with other cutaneous surfaces. We are unaware, however, of any comprehensive experimental studies which have examined the ability of the intraocular tissues, conjunctiva, or cornea to support this type of delayed hypersensitivity inflammation. Edmund[8] mentions a lack of corneal response to a contactant, having tested only two guinea pigs. It is known[9,10] that these ocular tissues will support the microbiallergic type of delayed response, typified by the tuberculin reaction, but their responses to well characterized simple chemicals in the experimental animal seems to be imperfectly defined.

Materials and Methods

Guinea pigs. Young adult albino guinea pigs, predominantly females, of the Hartley strain, weighing 400 to 600 gm., were used. They were fed Purina rabbit chow supplemented with fresh vegetables, with water ad lib. All eyes were examined for signs of abnormality before initiation of each experiment.

Antigens. The simple chemical contactant chosen for this study was 2, 4-dinitrofluorobenzene (DNFB). Egg albumin combines with DNFB to yield the dinitrobenzene-egg albumin conjugate (DNB-EA). This was prepared by a modification of the method of Sanger,[11] using an initial ratio of 20 moles of the dinitrobenzene derivative per mole of egg albumin. Bovine serum globulin conjugates (DNB-BSG) were prepared by the method of Eisen, Carsten and Belman,[12] using the sodium salt of 2,4-DNB-sulfonic acid, with an initial hapten : protein molar ratio of 94 : 1.

Sensitization. Contact sensitivity was achieved in the guinea pig as described by Eisen et al.,[13] employing a 1 : 100 saline dilution of a 20 mg./ml. stock solution of DNFB. Each animal received 0.1 ml. intradermally on five consecutive days. Anaphylactic sensitivity to the DNB grouping was effected by intraperitoneal injection of 1 mg. of either DNB-EA or DNB-BSG.

Challenge injections. A stock solution of 20 mg./ml. of DNFB in absolute ethanol was prepared, and dilutions of this for challenge were made in sterile isotonic saline on the day of use. Injections into the vitreous were made in 0.02 ml. volumes, while subconjunctival injections were of 0.05 ml. For topical challenge, an ointment of DNFB in white petrolatum was placed in the lower conjunctival sac and the lids manipulated gently to spread the ointment over the surface of the eye.

Since DNFB is somewhat toxic to normal tissues, it was necessary for each route of challenge to establish a subtoxic dose, so that any inflammation observed might be ascribed to hypersensitivity phenomena rather than to direct toxicity of the agent. Normal guinea pigs were exposed to varying dilutions of DNFB via each of the desired challenge routes and from these titrations a suitable subtoxic dose was chosen. For both the subconjunctival and intravitreal routes

this proved to be 0.5 mg./ml. The topical ointment contained 1 mg. DNFB per gram of petrolatum. Ocular challenge with DNB conjugates was made with a 5.0 mg./ml. solution of the protein in saline, employing 0.02 ml. for the intravitreal dose and 0.05 ml. for the subconjunctival injection.

Contact skin tests were performed by preparing a solution of DNFB in a 50-50 mixture of acetone and corn oil, at a concentration of 2.0 mg./ml. Two small drops of this solution were placed on the shaved dorsal skin of the guinea pig, and gently spread over a one square cm. area with a glass rod. After an interval of 44 hours, the area was depilated with a commercial depilatory agent and two hours after this the skin tests were read.

Intravenous challenge of guinea pigs with DNFB protein conjugates was made by incising the skin of the rear leg to expose the saphenous vein and injecting at least 1 mg. of conjugate through a 27-gauge needle.

Observations. Guinea pigs challenged into ocular tissues were generally observed at one to four, 24, and 48 hours following challenge and examined with the slit lamp and ophthalmoscope. Representative eyes were enucleated for histologic examination after challenge. These were fixed in aqueous formalin, embedded in paraffin, sectioned at five to seven microns thickness, and stained with hematoxylin and eosin.

Results

A. *Responses to DNFB of guinea pigs contact-sensitive to DNFB.* A number of animals were given a course of intradermal dinitrofluorobenzene (DNFB) injections to sensitize them to this simple chemical. The presence of contact sensitivity was established in each animal by skin testing with a topical application of the agent. While the degree of sensitivity varied from animal to animal, all of those tested gave definitely positive skin reactions consisting of varying degrees of erythema and induration.

1. *Intravitreal challenge.* DNFB-sensitive animals were challenged with the simple chemical alone into the vitreous. All 12 of the eyes injected failed to respond significantly to the allergen, although topical application of the same substance on the skin of these same animals provoked typical contact dermatitis. The only responses noted in these eyes were minimal reactions to the trauma of the injection, shared also by five control eyes in unsensitized animals.

2. *Subconjunctival challenge.* Five sensitized guinea pigs were challenged by subconjunctival injection into the superior bulbar region with a solution of DNFB. None of the injected eyes displayed a significant reaction apart from the minimal local response to the trauma of the injection. Control eyes injected in the same way showed similar minimal traumatic responses.

3. *Topical challenge.* 12 contact-sensitive guinea pigs were challenged with the simple chemical alone by applying into the lower cul de sac about 0.1 ml. of ointment. Nine of these eyes showed a clinically significant response to the challenge. At 24 hours, and also at 48 hours, a mild inflammation of the eyelids was noted in this reacting group, including erythema and often mild edema. Accompanying the lid response were varying degrees of conjunctival injection and edema in four of these animals. In five eyes, definite cloudiness of the cornea with mild limbic vascularization was observed accompanying the allergic blepharo-conjunctivitis. 12 nonsensitized control animals were challenged in the same manner with the DNFB ointment, and failed to show these changes.

B. *Response to DNB-protein conjugates of DNFB sensitized animals.* The sensitization of guinea pigs by intradermal administration of a simple chemical such as DNFB leads not only to the development of a delayed hypersensitivity state capable of supporting a contact dermatitis reaction, but also to the elaboration by the animal of demonstrable circulating antibody. As Eisen[7] showed, animals with antibodies specific for a simple chemical will respond with systemic anaphylactic shock to intravenous challenge with protein conjugates of the homologous

chemical. In the present study, we also found that conjugates of dinitrofluorobenzene with egg albumin, when injected intravenously into the DNFB-sensitized guinea pig, provoked in the five animals tested symptoms of profound anaphylactic shock which was fatal in four. Since antibody production to the chemical hapten and the resultant state of immediate hypersensitivity, may be responsible for a form of drug or chemical allergy, the responses of these animals to protein conjugates of the chemical were examined.

1. *Intravitreal challenge.* Guinea pigs sensitized with DNFB alone were challenged by intravitreal injections of 0.02 ml. of a 0.5 percent solution of DNB-EA conjugate. A number of these animals exhibited symptoms of moderate systemic anaphylactic shock, with snuffling, labored respiration and in a few instances involuntary urination. These responses generally were mild and never fatal. Eleven of the 12 animals responded with clinical signs of uveal inflammation after the intravitreal challenge. In three the reactions were rather mild. They all began within the first hours after challenge. In several, the response was characterized by a severe keratitis, with loss of epithelium and massive infiltration of corneal stroma by polymorphonuclear leukocytes, such as we have previously seen in studies on the Arthus response to protein antigens in the guinea pig eye.[10] In the present study, however, there was often a lesser involvement of the anterior uvea than we had observed previously. Those uveal responses seen were characterized by polymorphonuclear infiltrates typical of the ocular Arthus reaction, and two animals showed hemorrhage into the posterior vitreous in conjunction with a posterior segment inflammation.

2. *Subconjunctival challenge.* Eight eyes were challenged by subconjunctival injection of 0.05 ml. of a 0.5-percent solution of DNB-EA. Six of the injected animals reacted, two quite strongly. The animals reacting most intensely showed chemosis and exhibited a diffuse keratitis with marginal vascularization and cells in the anterior chamber. Although histologically the corneal infiltration by polymorphonuclear leukocytes extended to the center of the cornea, vascularization even at 48 hours was minimal, being confined to the periphery of the cornea. In conjunction with the conjunctival reactions there was often a mild diffuse polymorphonuclear infiltration of the ciliary body and adjacent choroid, while the iris in all cases remained essentially uninvolved.

C. *Response to DNB-protein conjugates of conjugate-sensitized animals.* The preceding experiments make it clear that the guinea pigs sensitized with DNFB alone develop not only contact sensitivity but also circulating antibody specific for the chemical hapten, that is, they have simultaneously immediate and delayed hypersensitivity. The demonstration by Eisen and coworkers[7] that DNB-protein conjugates are incapable of inducing contact sensitivity in the guinea pig allows us to prepare animals with only immediate hypersensitivity to the hapten, as a control for the previous experiments.

Guinea pigs were sensitized to dinitrobenzene by the intraperitoneal injection of one mg. of the protein conjugate, DNB-EA. Since this procedure produces not only antibodies against DNB, but also antibodies to egg albumin, challenge of these animals to study the DNB response is obviously not permissible using the same DNB-EA conjugate, for then any response might be attributable to the EA protein moiety. To obviate this difficulty, challenges with protein conjugate were performed with DNB coupled to bovine serum globulin (DNB-BSG); thus the only antigenic determinant common to both sensitizing and challenging substances would be the DNB. In accord with prior observations, animals sensitized to DNB-EA gave no contact dermatitis response to topically applied DNFB but could be sent into severe anaphylactic shock upon intravenous challenge with the heterologous DNB-BSG.

Following challenge with DNB-conjugate

into the vitreous of conjugate-sensitized guinea pigs, four of the five injected eyes reacted with clinically detectable uveitis. The reactions started within the first few hours. One animal killed at one to one and one-half hour showed histologically a hyperemia of the iris and ciliary body and marked hyperemia and a mild diffuse polymorphonuclear infiltrate in the choroid. At 24 hours, the remaining three reactors showed a mild iridocyclitis with cells in the anterior chamber and vitreous. The reactions subsided fairly rapidly, so that at 48 hours only one eye showed a continuing response. Four animals challenged by subconjunctival injection with the protein conjugate showed only an early mild conjunctival hyperemia and slight edema, which had subsided on examination at 24 hours. At this time, three of the eyes showed only minor marginal vascularization of the cornea adjacent to the injection site.

D. *Response to DNFB of DNB-conjugate sensitized animals.* Eisen and coworkers have shown[13] that following injection of DNFB, conjugation of the chemical with tissue proteins occurs. In animals with appreciable amounts of antibody specific for this haptenic grouping, anaphylactic mechanisms might result in the development of inflammatory responses to such a challenge injection.

Guinea pigs sensitized with DNB-EA were therefore challenged with the simple chemical alone by the several ocular routes. Following intravitreal challenge, seven of the 10 injected eyes showed an iridocyclitis. Five eyes showed only a mild involvement with cells in the anterior chamber and vitreous but without flare. In many of these animals the reaction appeared to intensify somewhat between 24 and 48 hours. The response in 10 eyes to subconjunctival challenge was only a mild conjunctival edema and hyperemia after four hours, which was thought not to differ significantly from the control eyes. At 24 hours, all eyes were negative. 13 animals were challenged by application of DNFB ointment into the conjunctival sac. Two of these showed a mild blepharoconjunctivitis in association with marked corneal edema, infiltration and central erosion. In addition, seven eyes showed a discrete focal punctate keratitis with some limbic vascularization after 24 hours.

Discussion

Guinea pigs sensitized by intradermal injections of DNFB readily develop contact sensitivity to this simple chemical, as manifested by typical contact dermatitis reactions to topical application of the chemical on the skin. Intravitreal injection of DNFB into these animals yielded no inflammatory response in those tested, suggesting that the uvea and retina are unable to support inflammation by a contact sensitivity mechanism. Similarly, contact sensitive animals tested by subconjunctival challenge with DNFB failed to respond with an allergic conjunctivitis. Topical challenge of the eye with DNFB in ointment gave positive, though mild, inflammatory reactions of the lids in nine of 12 hypersensitive guinea pigs. In some cases the response involved the neighboring conjunctiva as well as the lid.

The results of these experiments suggest that in the contact-sensitive guinea pig, the conjunctiva appears to be insusceptible to the *primary* action of the allergenic contactant. This is in accord with other evidence of the lack of primary reactivity of mucosal surfaces. While the very occasional response of the oral mucosa to a contactant in exquisitely hypersensitive individuals has been described,[14] this seems to be highly exceptional. Indeed, it seems to have been common practice among American Indians to chew the leaves of the poison ivy plant without ill effect, perhaps as a primitive attempt at desensitization. Sulzberger[15] has reported on the lack of oral and gastric mucosal response upon ingestion of a contact allergen by a sensitive patient, but significantly, there developed a perianal eczematous reaction to the allergen which had not been degraded in the intestine. Similarly, Jadassohn[16] reported

the use of intravaginal medication with iodoform in a patient contact-sensitive to this substance, without inflammation of the vulvar or vaginal mucosa. Accidental contact of the skin of the thigh with the material during treatment, however, yielded an acute contact dermatitis at this cutaneous site.

The ability of the conjunctiva to participate secondarily when the skin of the neighboring lid is responding to the chemical with allergic inflammation remains a possibility. The absence of delayed hypersensitivity reactions to simple chemicals on the part of the intraocular tissues is similarly in accord with prior experience, in which no other internal tissues were found to respond to the simple allergen with contact-type reactions.

Animals sensitized to DNFB are also found to produce circulating antibody specific for this chemical grouping and thus under favorable conditions might be expected to support hypersensitivity reactions of the anaphylactic type. Even better antibody response to a chemical hapten is elicited by immunizing the animal with heterologous protein conjugates of the chemical. Dinitrobenzene conjugates with egg albumin (DNB-EA) injected intravenously into DNFB or conjugate sensitized animals indeed resulted in severe anaphylactic shock. Even intravitreal and subconjunctival injection of the DNB-EA conjugate resulted in varying degrees of ocular inflammation in many of the eyes challenged. Since conjugates of simple chemicals with unrelated proteins have been shown to participate in immediate hypersensitivity reactions, these results in animals known to have antihapten antibody are not surprising.

Conjugates of DNFB with protein are known[7] not to confer contact sensitivity, so that it is clear that the conjunctival and uveal responses in conjugate-sensitized animals must necessarily be based upon an immediate hypersensitivity mechanism. In a similar way, those responses observed in conjugate-sensitized animals challenged with DNFB must be attributed to antibody-mediated hypersensitivity mechanisms. Animals so sensitized can be shown consistently to be free of contact sensitivity by skin testing but to have anti-DNB antibody. We must conclude that, as Eisen et al.[13] have shown, in vivo conjugation with protein occurs in this instance and that the conjugate then interacts with circulating antibody to set into motion the sequence of events leading to inflammation. Preliminary studies of corneal responses suggest that the latter mechanism may operate also in this tissue. The results relating to corneal responses to simple chemicals will be presented elsewhere.

The data presented in this paper suggest that the uveal tract, retina and conjunctiva of the sensitive guinea pig cannot respond to direct local challenge by the contact allergen, but that the conjunctiva may become involved secondarily to a contact dermatitis response of the eyelids. Caution must be exercised in generalizing from one species to another, especially in view of the demonstration[13] that man may be up to 100 times more sensitive than guinea pigs to certain simple compounds. However, these same results in man would not be surprising but rather very much in line with prior knowledge on the lack of reactivity of noncutaneous tissues to simple chemicals.

Summary

1. The immunologic reactions of ocular tissues in the guinea pig were examined with respect to mechanisms of the contact dermatitis type and of the anaphylactic type, employing the simple chemical 2,4-dinitrofluorobenzene and 2,4-dinitrobenzene conjugates with protein.

2. The data suggest that the uveal tract, retina and conjunctiva are unable to support a contact-type of inflammatory response to the allergenic chemical as a primary process, in accord with previous demonstrations that noncutaneous tissues are not generally able to respond with inflammation to challenge with the chemical contactant.

3. Guinea pigs which had produced anti-

bodies specific for the simple chemical were able to respond with anaphylactic-type ocular inflammation to challenge with protein conjugates of the chemical.

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DISCUSSION

DR. PHILLIPS THYGESON (San Francisco): I welcome the opportunity to discuss this paper, even though my interest in hypersensitivity has been primarily on the clinical side.

I recall that in the 1920's I wrote a thesis on the subject of hay fever and asthma at the time when all allergies were considered psychosomatic, and anybody interested in allergy was looked upon with great suspicion. Times have changed, and hypersensitivity is now the leading contender in disease production. I am interested particularly in the allergy of infection since it seems to appear that the delayed reaction is responsible for many of the clinical signs of infections. I have followed to a certain extent the literature on the immunology of the eye, and certainly have been struck by the scarcity of experimental studies dealing with the contact mechanism; so I think we can all welcome the very well done series of studies and particularly this last one of the series dealing with the contact mechanism.

In this paper, the authors mentioned the similarities between the tuberculin reaction and the contact reaction. However, they did not stress the differences. It seemed to me that the two must be essentially the same reaction, differing only in the type of antigen, the tuberculin antigen being a protein while a simple chemical requires a conjugation with body proteins to become antigenic. I think many of the differences can be accounted for on this basis.

I was interested in the question of whether or not the skin was uniquely concerned in contact reactions. Certainly clinically we do have a conjunctivitis as a part of the contact mechanism. I think particularly of atropine dermatitis of the lids with conjunctivitis. The question arises as to whether the conjunctivitis here is an expression of the increased sensitivity of the human over the guinea pig, allowing the mucous membrane to participate in the contact mechanism, or whether the conjunctivitis is simply a spill-over from the lid as a secondary mechanism, or whether it reflects an immediate sensitivity reaction going along simultaneously with the contact mechanism.

In this respect I bring up the question of the significance of eosinophilia. I have been told by dermatologists that in the contact mechanism one should not expect an eosinophilia. However, in

practice, in studying these atropine or other drug reactions, more than half of the cases do show a conjunctival eosinophilia, which would more or less suggest that the conjunctival reaction may be an immediate reaction tied in with the delayed reaction.

In connection with drug reactions it is of interest to consider a very marked follicular hypertrophy of the conjunctiva develops in the absence of eosinophilia and in the absence of skin reaction; I presume this would indicate a toxic rather than a hypersensitivity mechanism.

In clinical ophthalmology we are impressed with the variety of sensitivity reactions. Phlyctenosis is one example, where the limbus is the primary shock tissue with spill-over onto the conjunctiva or the cornea as the case may be; and then hay fever conjunctivitis, certainly an immediate reaction, with a non-specific conjunctival reaction; and then vernal catarrh, in which there is an upper tarsal or a limbic involvement or a combination of the two. Vernal must be an immediate type of reaction along with hay fever conjunctivitis, in contrast to phlyctenosis, a delayed reaction.

The time is certainly ripe for further experimental studies on ocular hypersensitivity, and I think we are very lucky to have this excellent immunology-pathology team available to continue these studies in a long-neglected field.

DR. SEYMOUR P. HALBERT (New York): In view of the fact that the simple chemical compounds do not seem to do this type of damage, and in view of the comments Dr. Silverstein has made and for which I believe there is abundant evidence that the simple chemicals apparently seem to have to combine with certain substances in the skin, and in view of the specificity of the skin in this respect, I wonder if perhaps the substrate with which the simple chemical combines might be related to the keratins.

If a complex of the simple chemical and perhaps the keratin of the skin is required to induce the reaction, I wonder if such a complex might induce the reaction on the conjunctiva or the other mucous membranes.

I wonder if Dr. Silverstein has done any studies of this sort.

DR. PRASANTA K. BASU (Toronto): I have a brief question to ask Dr. Silverstein about the cytology of these reactions. If it is immediate, you would expect more polymorphonuclear reaction. If it is a delayed type, there would be more a muscle type of reaction. I would like to hear some discussion of the cytologic studies.

DR. A. M. SILVERSTEIN (Closing): I would like to thank the discussers. I think that if any point should be made about this work, it is that, as I suppose one generally says, this is preliminary. It is our first crack at this type of thing, and I don't think one should come away from this presentation under the impression that we are trying to say that the skin is the only tissue that can react. I do think it would be fair to come away with the impression that we think now that the evidence supports this point of view.

I believe, however, that the species difference between the guinea pig and the human is sufficient reason to leave the question in one's mind. I would also be remiss if I did not state that occasionally it has been observed in the individual exceptionally equisitely hypersensitive to poison ivy that the oral administration of poison ivy extract did give rise indeed to a stomatitis. However, whether this is really an immunologic reaction or a toxic reaction, one cannot say.

With respect to the eosinophilia; I have seen in the literature the comment that the presence of the eosinophiles is characteristic of a contact conjunctivitis and, indeed, is to be looked for in making such a diagnosis; and yet this goes against statements by experimentalists in this field, as Dr. Thygeson pointed out, and by clinical dermatologists, who tell me that the eosinophil is not characteristic at least of the cutaneous lesion of contact dermatitis.

With respect to Dr. Halbert's question on the reaction to conjugates of the simple chemicals with skin substances such as keratin; we have not tried this, although we intend to. I might point out that this has generally been impossible for other experimental workers in this field to do. That is, one assumes that one needs a conjugate of the simple chemical with some skin component to stimulate the reaction, and one would think that the in vitro preparation of such a conjugate would suffice to give an antigen which then in any tissue could stimulate the contact hypersensitivity mechanism. However, this has turned out not to be so, for reasons unknown.

With respect to the question about the cytology of this lesion; we haven't gone too far into it. I might mention merely that other investigators, again working on the cytology of this lesion in the skin, have shown that it is indeed similar to the mild tuberculin reaction and to other delayed hypersensitivity reactions which do not go to the extreme of tissue destruction, in that it is characterized by the familiar perivascular infiltrate of round cells.

We have yet, I would emphasize, a lot to do in studying the reactions of ocular tissues elicitable by this immunologic mechanism, and a careful study of the histopathology is one of the things that we plan in the future to examine more extensively.

THE RAPID AND CONVENIENT DETECTION OF TOXOPLASMA ANTIBODIES USING FORMALDEHYDE-TREATED HUMAN ERYTHROCYTES*

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In 1948, Sabin and Feldman described the methylene blue dye test for the detection of antibodies to Toxoplasma.[1] Through this test, knowledge of the manifestations of the infection with Toxoplasma has been greatly advanced. The performance of this test, however, requires the use of freshly obtained and potentially dangerous live organisms which, after staining, must be examined microscopically and laboriously counted to determine the proportion of organisms in each serum dilution which is unstained. Although other serologic tests have been proposed,[2,3,4] in general, even those from which antibody titers can be obtained, appear to be less sensitive than the dye test and not comparable to it.

In 1957, Jacobs and Lunde first reported a hemagglutination test for toxoplasmosis.[5] With proper standardization the titers are comparable to the dye test titers, although in some cases of acute infection hemagglutination antibody appears a few days after the dye test antibody is detected.[6] This new test eliminates the need for freshly obtained live organisms, as the test is done with a nonviable saline lysate as sensitizing antigen. This antigen, unlike the organisms, is not only safe, but also is usable after storage in the frozen or lyophilized state.[6] Despite its many advantages, however, the hemagglutination test requires red blood cells which can be kept only a limited time either fresh or sensitized. Since sensitized fresh erythrocytes can be stored for about 15 days, the preparation of sensitized cells is laborious unless large numbers of sera are to be tested in a short time.

Flick, in 1948, discovered that erythrocytes treated with formaldehyde could be preserved for long periods of time.[7] Since such erythrocytes can be frozen or lyophilized, it seemed possible that formaldehyde treated cells might be preserved so as to keep a constant supply of cells available. In addition, the preservation of such cells after sensitization might permit serum dilutions to be tested simply by adding aliquots of this previously preserved reagent. The studies to be reported concern the suitability of formaldehyde treated preserved cells in the hemagglutination test for toxoplasmosis.

METHODS AND MATERIALS

Several techniques of formaldehyde treatment were found to be satisfactory, as long as formaldehyde was added slowly to cells that were constantly kept agitated. The gradual exposure of most of the cells used in this study was accomplished by placing the formaldehyde in a dialysis bag in a beaker of constantly agitated cells. The volume of formaldehyde used was one-quarter the volume of the 12.5 percent suspension of cells. After one day, the contents of the bag were poured into the cells and after two more days the cells were washed seven to 10 times in saline and could then be used. This is similar to techniques previously described.[8] Although for most experiments type O RH negative erythrocytes were used, erythrocytes treated with formaldehyde do not agglutinate with the standard blood type agglutinins and any type cells appear to be satisfactory. Thus, apparently, any "out of date" bank blood can be formalinized and used.

The tanning and sensitization of the

* From the Uveitis Laboratory of the Howe Laboratory of Ophthalmology. This work was supported by U. S. Public Health Service Grant B 2036 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health.

treated erythrocytes was only slightly modified from the method described for fresh erythrocytes.[5] In the preliminary tanning, a 5 percent by volume suspension of cells was exposed to an equal volume of 1/40,000 tannic acid at 37°C for 90 minutes. The cells were then washed once with pH 7.2 phosphate-buffered saline and were diluted to their original volume in normal saline.

In order to sensitize these cells, an equal volume of antigen, previously diluted in pH 6.4 phosphate-buffered saline and standardized as to concentration with either fresh or formaldehyde treated cells against sera of known titer, was incubated at room temperature for 30 minutes with the constantly agitated tanned cells. The cells were washed once with twice their volume of 1.0 percent normal rabbit serum in saline and were resuspended in the 1.0 percent rabbit serum to their initial volume. For testing serum dilutions 0.05 ml. of cell suspension were added to 0.5 ml. of the diluted serum in a standard hemagglutination tube.

Tests were generally read after two hours of incubation at 37°C, although occasionally the results were more clearcut if the cells were permitted to stand at room temperature overnight (fig. 1).

Some cells at each stage were stored by simple refrigeration between 0.0°C and 5.0°C. Some were quick frozen in a dry ice ethanol mixture and kept at minus 15°C, and some were quick frozen and then lyophilized.

Results

Hemagglutination tests with formaldehyde treated erythrocytes were done on 75 sera which had been tested by hemagglutination with fresh cells and by the Toxoplasma dye test.* In all cases, the hemagglutination titer was within one dilution of the dye test titer and in all cases the titers of fresh and formaldehyde treated cells were identical. In the hemagglutination test, the buttons of non-agglutinated cells were sometimes not as sharply defined with the treated cells as with the fresh cells, but end-points were easily read and the results were identical. In addition, the amount of antigen required to sensitize the fresh and the treated cells was identical. Antigen dilutions standardized with either type of cells could be used for the other.

The buttons of cells seen in the absence of antibodies were smallest and most sharply defined when the sera to be tested were diluted with 1.0 percent dye test negative rabbit serum in saline. The substitution of 1.0 percent normal human serum as diluent prevented the cells from settling properly. One-half percent human serum was a satisfactory diluent, though inferior to the rabbit serum, and permitted end-points to be determined after one hour of incubation. Although the use of saline without any serum permitted

* The initial determination of the suitability of formaldehyde treated erythrocytes was done with Dr. Leon Jacobs and Mr. Milford Lunde.[5]

Fig. 1 (Maloney and Kaufman). Hemagglutination test with formaldehyde treated erythrocytes. Concentrated antibody-containing sera produce larger diffuse patterns of cells as seen on the left. As the antibodies are diluted out, the cells settle in small buttons. In this case the change in pattern occurs after the second tube and the dilution of serum in this tube, which is 1/64, is taken as the titer.

TABLE 1

PRESERVATION OF FORMALDEHYDE TREATED ERYTHROCYTES

Method of Storage	Formaldehyde Treated Cells	Time of Preservation
Refrigerated 0°–5° C.	Formaldehyde treated only	3+ months
	Tannic acid treated	3+ months
	Sensitized	6+ weeks
Lyophilized	Formaldehyde treated only	2+ months
	Tannic acid treated	3+ months
	Sensitized	2 months
Frozen, −15°C.	Formaldehyde treated only	6 months
	Tannic acid treated	3+ months
	Sensitized	3 months

the satisfactory determination of titers, the end-points were not as easily read.

After refrigeration for three months at 0.0°C to 5.0°C, the cells that were formaldehyde treated, but not tanned or sensitized, reacted well in the hemagglutination test. Similarly, formalinized erythrocytes that had been tannic acid treated were excellent for use in the hemagglutination test after three months of storage. Samples of sensitized cells kept for six weeks under refrigeration were satisfactory, but the tests were more difficult to read (fig. 2). All cells that were refrigerated were satisfactory reagents at the end of the testing period and the storage times cited in no way represent a maximum.

Lyophilized erythrocytes could be preserved after formaldehyde treatment alone for at least two months. When the formaldehyde treated cells were tannic acid treated before lyophilization they were well preserved after three months and still excellent for testing. After sensitization, however, the frozen and dried preparations gave good results for only about two months and were often not satisfactory after this time.

Frozen formaldehyde treated cells were perfectly satisfactory after at least six months of storage. One phlebotomy provided us with a source of cells for some time. When tannic acid treated formalinized erythrocytes were frozen, excellent results were obtained for at least three months. This does not represent a maximum storage time. Cells frozen after being sensitized provided good results for at least three months but the tests were not quite as easily read as when the frozen tannic acid treated cells were sensitized immediately before use.

In our laboratory, for routine use, we prefer to use tannic acid treated formalinized erythrocytes either frozen or just refrigerated. They are incubated at room temperature for 30 minutes with the stored antigen and are then added to the serum dilutions. Results are read after two hours of incubation.

Discussion

To simplify and render safe the investigation of antibodies to Toxoplasma, a test should not require live organisms. The reagents should be stable and the performance of the test should be simple and not overly time consuming. The test should be sensitive, should provide a quantitation of antibody titer, should be reproducible and, ideally, should be comparable to other standard procedures.

Although other tests have been developed,[2, 3, 4, 9] the use of formaldehyde treated cells in the well standardized hemagglutination test to measure Toxoplasma antibodies appears nearly ideal. The cells are simple to prepare; they are merely exposed to formaldehyde and washed. Any type of blood is usable. They can be preserved to supply a source of cells for some time for use with a sensitizing antigen which is noninfectious and is also stable. In addition, once the cells are treated with tannic acid or sensitized, storage of the erythrocytes is possible and in testing a serum dilution only the addition of this previously prepared complex and a brief incubation are necessary. The test is exceedingly simple to read. Even if few sera are to be tested the procedure appears sufficiently easy and reliable to be practical.

In the hemagglutination test with formaldehyde treated erythrocytes, regardless of the age of the cells, the titers did not change.

Fig. 2 (Maloney and Kaufman). Row 1 is negative controls. Row 3 is a hemagglutination test with fresh erythrocytes. In the other rows formaldehyde treated erythrocytes are used. Row 2 is formaldehyde treated only preserved by refrigeration 3 months after preparation. Row 5 is tannic acid treated preserved by lyophilization 3 months after preparation. The titers of rows 2, 3 and 5 are sharp and easily read. Row 4, however, illustrates the appearance of cells that are sensitized and preserved for 6 weeks by refrigeration. Under these conditions the buttons of cells seen with highly diluted serum are larger and the test is more difficult to read, although the titre is unchanged.

Results in all the cases were identical to those obtained with the standardized hemagglutination test employing fresh erythrocytes and were within one tube dilution of the dye test results. In some patients that have been acutely infected, the hemagglutination antibodies may appear a few days later than dye test antibodies, but in general hemagglutination titers and dye test titers are nearly the same.[5]

Summary

Toxoplasma hemagglutination antibody titers are comparable to those of the more

laborious and dangerous Toxoplasma dye test, but can be determined easily and do not require the contact with live organisms. The ability to preserve cells after they have been tannic acid treated or sensitized makes practical the determination of Toxoplasma antibody titers of sera over a period of months from a single supply of prepared cells. The determination of Toxoplasma antibody titers by this method appears to be simple, rapid, sensitive and safe.

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Discussion

Dr. Seymour P. Halbert (New York): In discussing this excellent paper by Mrs. Maloney and Dr. Kaufman, I felt it might be appropriate to put their observation into proper perspective. These observations relate to diagnostic methods in infectious disease. How do we diagnose infectious disease?

There are two general ways, and the first and probably the best is to find actual evidence for the presence of the infectious agent. The diagnosis can usually be made during the acute phase of the illness. One can do this by isolating the organism, first, which is often difficult and sometimes impossible because of lack of access to the material.

The second is by obtaining evidence for the presence of the infectious agent by specific staining with fluorescent antibody, a technique which is going to be more and more widely used, one can predict, within the next five years or so.

The second general way to diagnose infectious disease is to search for an antibody to the specific infecting agent, and this type of observation of course is retrospective. In acute illnesses one should obtain both serum from the acute phase of the illness as well as during convalescence—paired serum—and a comparison of the two sera with respect to a specific antibody rise is most essential in making a diagnosis.

The antibody reactions that are used include agglutination reactions, complement fixation reactions, agar precipitin reactions, animal protection reactions, and so on. But by far, apparently, the most sensitive of all methods for protection of antibody in vivo are those used by Dr. Kaufman and others—antigen-coated red cells, the antigen hemagglutination test. It is not uncommon with potent experimental serum to be able to dilute the serum one to several million and still get good hemagglutination reaction.

The hemagglutination method, using tanned red cells as described in these studies by Dr. Kaufman, is usually time-consuming and somewhat tedious. Often antigens are not accessible to other laboratories, and there are storage problems, and so on.

It has been shown here by the study of Mrs. Maloney and Dr. Kaufman that some of these problems can to a large extent be avoided. The hemagglutination method for the detection of antibodies can be readily made available to even the smallest laboratory. Incidentally, Dr. Park of Columbia University has also been doing such studies concurrently and has arrived at similar conclusions.

One may look forward to the day when a central source of supply or even biological laboratories may supply fixed Toxoplasma coated red cells in the lyophilized state for use in assay in the smallest and most remote diagnostic hospital laboratory. It will then be necessary only to re-suspend the fixed antigen-coated cells and assay the serum.

In addition, these observations open up the possibility of using similarly prepared fixed red cells for diagnosis of many other infectious diseases.

Dr. Park has already prepared stable, formalin-fixed tuberculin-coated red cells and has found that these agglutinate in much the same way after lyophilization as do fresh tuberculin-coated red cells with sera from tuberculous patients, and he has started similar studies with adenovirus materials and has obtained some promising results indicating that even in the field of virus diagnosis, which will be so important in ophthalmology, these methods may show a great deal of promise.

It seems to me it is still best to diagnose infectious disease by detecting the actual presence of the micro-organism or some product of it. The hemagglutination test can be adapted for this by the so-called hemagglutination inhibition test. For example, using Toxoplasma-coated red cells and the smallest amount of known antibody needed to cause hemagglutination. If one first adds to the antibody a minute amount of Toxoplasma antigen, binding occurs and then the bound antibody can no longer cause hemagglutination. This is hemagglutination inhibition. One can therefore use this extremely and extraordinarily sensitive method to attempt to detect specific Toxoplasma antigens from patient materials. For example, using aqueous humor and thereby convincingly demonstrating the presence of the Toxoplasma products in the eye, perhaps.

I would like to ask Dr. Kaufman if he has applied this modification of the hemagglutination inhibition test to see whether he could detect such Toxoplasma antigens in experimentally infected antigens in experimentally infected animals or patients.

Dr. Herbert E. Kaufman (closing): I would like to thank Dr. Halbert for his very stimulating and perceptive discussion.

His comments about the titers and the sensitivity of the hemagglutination test are correct. It is possible to increase the sensitivity of a hemagglutination test by increasing the amount of the antigen with which the cells are sensitized. The titers of the hemagglutination test can be increased and the sensitivity can be made greater, for example, than that of the dye test.

We have standardized our antigen so that our hemagglutination titers run parallel to the dye test titers. This procedure was described by Jacobs and Lunde and seems to be most workable.

The possibility that a central source could supply a formalinized red cell reagent which could be added to the serum in question sounds appealing. If the reagent were added to the serum and incubated for two hours the antibody titer could be determined simply by examining the bottoms of the tubes.

We do many determinations of antibody titers and a practical method is extremely important for us. In an attempt to develop a relatively simple test, we have tried many precipitin methods, various fluorescence and fluorescence inhibition methods, and have tried bentonite as well as other substances as possible vehicles for an agglutination test. Although many of these methods work, we feel from our experience that a test using formalinized human erythrocytes is the easiest.

Human blood is everywhere. The formalinized cells are easily stored. It appears to make no difference what type of cells we use—I would think that cells from various animals and all humans would be satisfactory. Just as the type of cell seems unimportant the method of formaldehyde treatment of the cells can be varied as long as the cells do not tend to agglutinate spontaneously. Jacobs reports that the hemagglutination antigen is stable. Although we have done no studies of antigen stability, we keep the tannic acid cells and incubate them with antigen for half an hour before the test, and we feel that this gives us the best end points.

The idea of a hemagglutination inhibition test is fascinating. We have had no experience with this. It would be wonderful if this could be applied to the eye, if, in fact, products of Toxoplasmas could positively be identified in the eye.

STEADY-STATE PRESSURE FLOW RELATIONSHIPS IN THE LIVING AND DEAD EYE OF THE CAT*

Maurice E. Langham, Ph.D.
Baltimore, Maryland

Introduction

The reaction of the eye to changes in pressure has recently been studied by an analysis of the relationship between the steady-state intraocular pressure and varied rates of flow of fluid through the anterior chambers of living and dead eyes of rabbits (Langham 1958; 1959b). It was found that the intraocular dynamics altered with pressure in a manner that tended to minimize changes in the psysiologic steady state pressure in the eye. These homeostatic responses were found to be dependent on an intact sympathetic nerve supply to the eye and supported the hypothesis that the regulation of the intraocular pressure is based on responses medi-

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ated by a neural reflex. Concepts of this nature have been under intensive investigation by several groups of workers in recent years and experimental evidence of the presence of pressure receptors in the eye has been given by von Sallmann, Fuortes, Macri and Grimes (1958) and Perkins (1959). These investigators observed a synchronous relationship between the afferent electrical activity in the long ciliary branches of the ophthalmic division of the fifth nerve and pressure changes in the eyes of cats.

The purpose of this study has been to establish the effect of pressure on the intraocular dynamics of the cat's eye. The influence of varied rates of flow of fluid through the anterior chamber on the intraocular pressure has been determined and the results compared to those found previously in similar experiments on rabbits.

Methods

Male and female cats weighing between 1.8 and 4.4 kg. were used in this study. Animals were anesthetized with Urethane (0.8 to 1.75 g./kg. injected intraperitoneally).

The intraocular and blood pressures were recorded with the Sanborn Instruments model 267 B transducers, model 150-1100 carrier preamplifiers and the model 150 M rectilinear pen recorder.

The experimental procedure used in the infusion studies was the same as that described in a previous paper (Langham 1959b). The infusion apparatus was designed to give exact and readily reproducible rates of infusion of fluid against a recipient pressure of at least 200 mm. Hg. (fig. 1). The gears of this machine were driven by the movement of a friction drive plate which in turn was driven by a revolving rubber wheel connected to the axle of a synchronous electric motor. The electric motor could be moved away from the drive plate by means of a cam to facilitate the rapid and smooth interchange of the gears. Eight gears enabled the speed of injection to be varied over a range of 20:1 and the rates of injection could be further modified by the use of different size syringes.

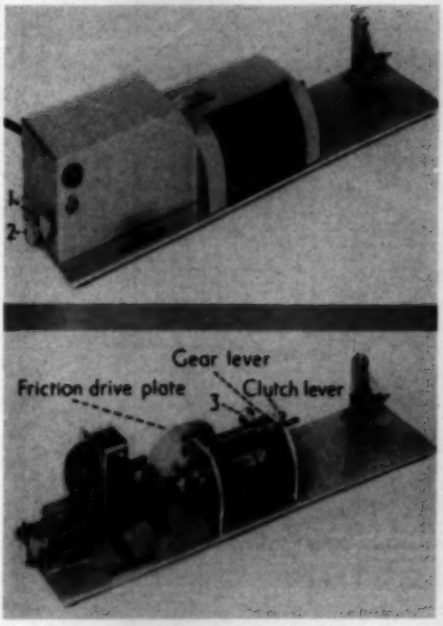

Fig. 1 (Langham). The microinjection machine. 1 indicates the on-off cam for disconnection of the motor from the drive plate; 2 indicates the rod for varying speed of friction drive plate; 3 indicates the handle for manual movement of the piston moving the plunger of the syringe.

The protein concentration in samples of aqueous humour was determined by a micronephelometric technique.

Results

The mean intraocular pressure of 57 eyes of 36 adult cats, anesthetized with Urethane, was 20.1 ± 0.37 mm. Hg. (Arithmetic mean ± the standard error of the mean). The corresponding mean pressure in the femoral arteries of the same group of animals was 119 ± 3.1 mm. Hg. The weight of these animals ranged from 1.8 to 4.4 kg. and no correlation was found between intraocular pressure and body weight.

The pressures in the eyes of individual cats were very similar. In 20 animals the mean intraocular pressures in the left and

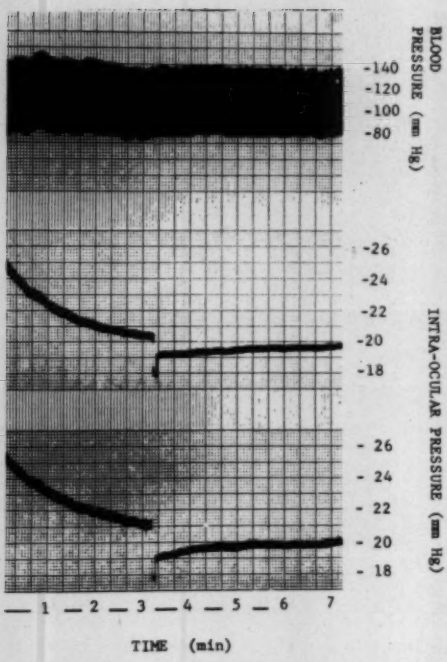

Fig. 2 (Langham). The technique for measurement of the steady state intraocular pressure. The upper curve shows the femoral blood pressure and the two lower curves, the intraocular pressures in the two eyes. The record shows the two convergent pressure curves obtained when the intraocular pressure was brought above and then below its equilibrium value.

right eyes were 20.6 ± 0.56 and 20.1 ± 0.62 mm. Hg. respectively and the mean pressure difference in pairs of eyes was 0.45 ± 0.15 mm. Hg.

These pressure measurements were made immediately after insertion of the recording needle into the anterior chamber, using the technique illustrated in Figure 2. The initial intraocular pressure was raised to 25 mm. Hg. in all animals by connection of the anterior chamber via the recording needle to a pressure reservoir of saline phosphate, set at a height of 34 cm. with respect to the eye. Approximately 20 seconds after the needle had been inserted into the eye, the tap to the reservoir was turned off and the pressure in the eye began to fall toward its equilibrium value. The pressure decay was recorded for approximately two minutes and then the intraocular pressure was decreased to a value below its equilibrium value by connecting the eye momentarily to the saline reservoir, set at a new level. In this way the steady state pressure was determined from the convergence of the descending and ascending pressure curves. The equilibrium pressure determined in this manner agreed closely with the value obtained by allowing the intraocular pressure to fall continuously to its equilibrium value. This latter type of observation took 10 to 15 minutes compared to the period of three to four minutes needed to obtain the two curves of convergence. By the application of this rapid technique in all the proceeding infusion studies experiments on individual eyes were completed within 30 to 45 minutes after insertion of the needle into the eye. After this interval of time there is a marked tendency for the normal intraocular pressure to decrease and the permeability of the blood aqueous barrier to increase. Similar permeability changes were found in experiments on rabbits (Langham 1959b).

The result of a typical infusion study on an anesthetized cat is recorded in Figure 3a. The mean femoral blood pressure of this animal remained constant throughout the experiment at a mean value of 135 mm. Hg. The intraocular pressure was recorded and then infusions were made in the order 4.5, 7.5, 6.0, 4.5, 2.8 and 0 μl. min.$^{-1}$. The intraocular pressure at the end of the experiment was 1 mm. Hg. below that at the beginning. The concentration of protein in the aqueous humour removed at the end of the experiment was 18 mg. percent which is within the range of values found in normal cats' eyes. Thus in a control group of six cats the protein concentration of the aqueous humours varied from two to 22 mg. percent.

The variation of results between different cats is shown in Figure 3. In all, perfusion studies were made on 32 eyes from 16 cats and the analysis of the results on 13 of these animals is recorded in Table 1 and Figure 4.

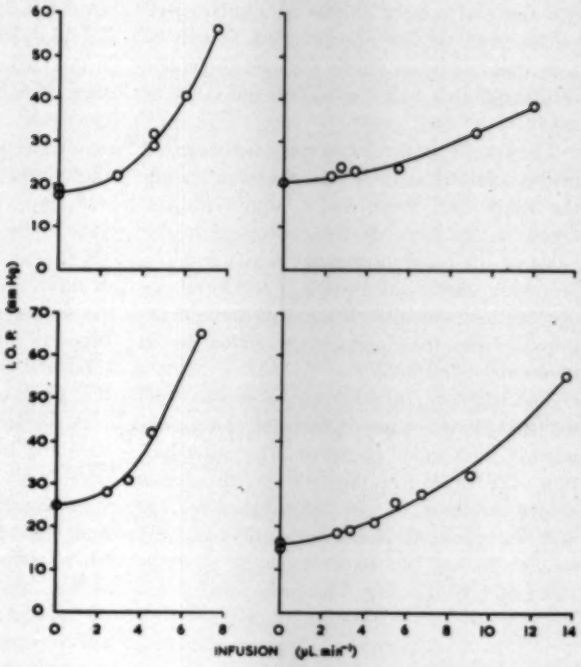

Fig. 3 (Langham). Relationship between steady state intraocular pressure and infusion rates in cats anesthetized with Urethane.

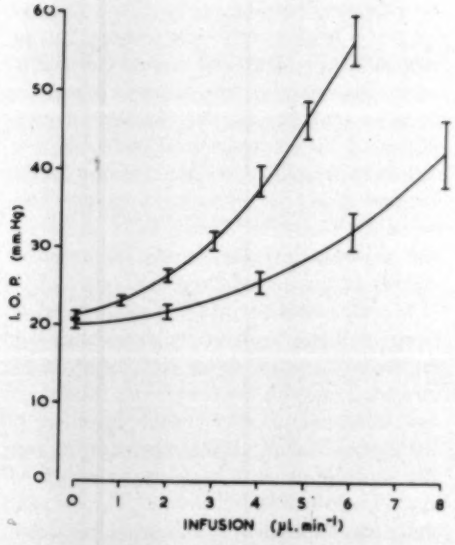

Fig. 4 (Langham). Comparison of the mean pressure/infusion relationship between the eyes of rabbits (upper curve) and cats (lower curve). The curve for rabbits was taken from a previous study based on 26 normal eyes (Langham 1959b) while the curve for cats was based on results recorded in Table I. Each bar represents the mean pressure ± standard error.

TABLE 1

MEAN INTRAOCULAR PRESSURE FOR DIFFERENT RATES OF INFUSION OF SALINE PHOSPHATE (PH 7.4) INTO THE ANTERIOR CHAMBER OF CATS ANESTHETIZED WITH URETHANE

Infusion Rate (µl. min.$^{-1}$)	Steady State Intraocular Pressure (mm.Hg.)
0.0	20.0±0.79 (13)
2.0	21.7±0.87 (13)
4.0	25.5±1.36 (13)
6.0	32.0±2.48 (13)
8.0	41.9±4.21 (13)

For comparison, the results of similar studies on adult rabbits are included. It will be seen that the mean curve for cats is similar in shape to that found in rabbits but is not as steep.

The results on three cats were not included in this analysis since it was observed during the study that there was a significant decrease in the slope of the curve and in the value of the basal intraocular pressure. Consequently, there was a marked scatter of the observations; samples of aqueous humour removed from these eyes were found to be moderately plasmoid.

The pressure/infusion curve on one animal that showed this marked decrease in the normal intraocular pressure after introduction of a needle into the anterior chamber is shown in Figure 5. The cat weighed 3.5 kg. and the femoral blood pressure remained steady during the experiment at a mean value of 100 mm. Hg. The intraocular pressure was determined and then the infusions 7.5, 15.0, 10.0, 5.0 and 0 µl. min^{-1} were made in this order. The initial and final pressures were 18.5 mm. Hg. and 12.0 mm. Hg. respectively. The intraocular pressure remained at 12 mm. Hg. during the following 15 minutes and then a further infusion study was made with infusion rates up to 60 µl. $min.^{-1}$ The second eye showed a similar instability and the final pressure/infusion curve in this eye agreed very closely with that in the first eye. It was noted (fig. 5) that the slope of this curve was approximately constant and equivalent to an outflow facility of 1.93 µl. $(mm. Hg.)^{-1}$ min^{-1}.

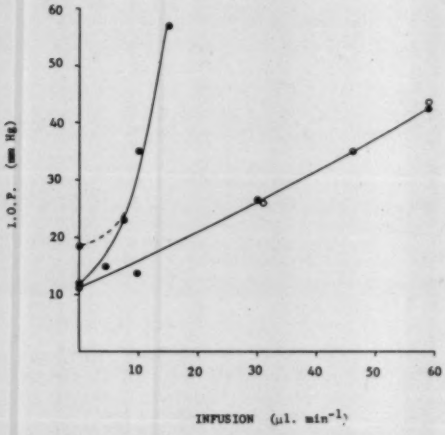

Fig. 5 (Langham). The results of an infusion study on 'unstable' eyes of an anesthetized cat. The initial intraocular pressure in each eye was 18.5 mm. Hg. The solid circles represent the observations made on one eye and the lower curve shows the final values found in the eye. The recording needle remained in the second eye 45 minutes before commencement of the experiment, during which time the intraocular pressure fell to 12 mm. Hg. Subsequently, a pressure infusion study was made and the results (O————O) agreed closely with the final curve on the first eye.

A similar decrease in the intraocular pressure and in the slope of the pressure/infusion curve appeared to be a characteristic response of all normal eyes when allowed to remain under these experimental conditions for a sufficient time. Thus, in four eyes a second series of infusion studies was made 20 to 30 minutes after completion of the first and in each case there was a significant decrease in the eye pressure and in the slope of the pressure/infusion curve. This was not due to a leak through the cornea for no fluorescein percolated out around the recording needle. For this test, fluorescein was introduced into the anterior chamber at completion of the experiment. The blood pressure of these animals remained steady during this period and the untouched eyes were subsequently found to have similar pressures and pressure/infusion curves as recorded initially in the contralateral eye.

A comparison of infusion studies on the living and dead eyes of the same animal is shown in Figure 6. After completion of the infusion study on the living eye the animal was killed by an intravenous injection of Nembutal. The intraocular pressure fell rapidly and approached a steady value of 8.0 mm. Hg. in five to 10 minutes. The infusion study was then made 15 minutes after death. It was found that the curve in the dead eye was approximately linear. Similar results were found in two other cats. Figure 6 also

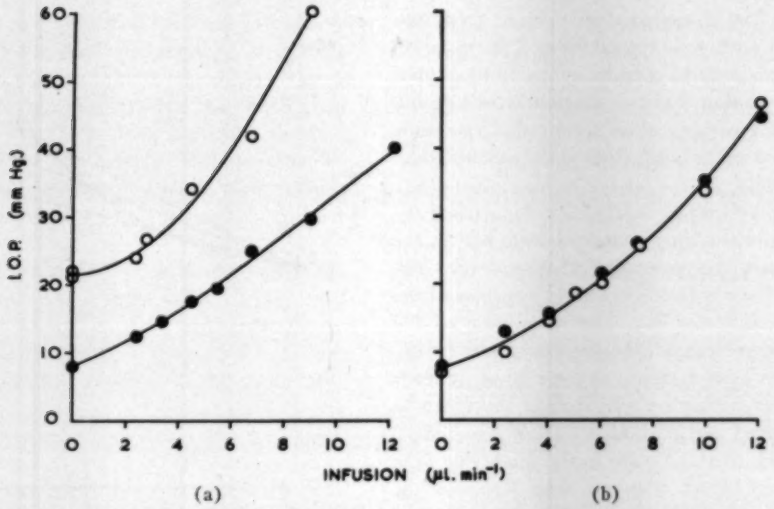

Fig. 6 (Langham). Relationship between steady state intraocular pressure and infusion rates in eyes after death. (a) Typical record of results on the living (○——○) and the dead eye (●——●), of a cat. (b) Results on a pair of enucleated eyes; studies on each eye were started 20 minutes after enucleation from the living animal and were made at room temperature.

includes the results of infusion studies on a pair of eyes freshly enucleated from an individual cat. Again the relationship between infusion rates and pressure was found to be approximately linear for pressures ranging from 10 to 35 mm. Hg. Three other freshly enucleated eyes gave similar results. The mean intraocular pressure in eight cats 15 minutes after death was 6.26 ± 0.56 mm. Hg.

Discussion

The observed relationship between the steady state intraocular pressure and different rates of infusion of saline phosphate into the anterior chambers of living and dead eyes of cats is qualitatively similar to that found in a previous study on rabbits (Langham 1959b). There are, however, certain quantitative differences between the results on the two species which merit discussion.

A comparison of the mean pressure/infusion relationships in cats and rabbits (Fig. 4) indicates that the mean intraocular pressures agree closely but that larger infusions of saline phosphate are required to raise the pressure in cats. To account for this difference it is helpful to compare the intraocular dynamics in the two species, using the basic steady state hydrodynamic equation of the eye, namely, that the rate of outflow F (μl. min^{-1}) = $(P_o - P_v)$ C where P_o and P_v are the pressures in the anterior chamber and episcleral veins respectively, and C is the outflow facility (μl. (mm. Hg.)$^{-1}$ min^{-1}). In normal cats the rate of flow of the aqueous humour has been reported to lie between 1.0 and 1.5 percent min^{-1} (Langham 1951; Becker & Constant 1956), a value similar to that in rabbits (Kinsey & Barany 1949). The absolute rate of flow must, however, be very different in the two species since the volume of the aqueous humour in the cat is approximately three times greater than in rabbits. In this study the mean volume of aqueous humour was 800 μl. which means that the value of F was 8 to 12 μl. min^{-1}. By way of comparison, the absolute rate of flow in rabbits is believed to be 2 to 4 μl. min^{-1}. Thus, while the intraocular pressures in the two species are the same, the rate of outflow

in the cat is approximately three times the rate of outflow in the rabbit and, parri passu, the resistance to outflow is one third of that in the rabbit. This conclusion is in keeping with the present observations that the slope of the pressure/infusion curve was less than in rabbits.

It is difficult to account for the marked decrease in the intraocular pressure and in the slope of the pressure/infusion curve observed one to two hours after insertion of the needle into the eye. The magnitude of the change in individual eyes could not be explained by a decrease in the rate of aqueous humour formation and it must therefore be concluded that part, if not all of the change, was due to an increase in the facility of fluid drainage from the eye. The slope of the modified pressure/infusion curve appeared to be approximately constant and similar in value to the outflow facility in the living and dead eyes of cats reported by Becker and Constant (1956). These investigators studied the rate of outflow of fluid into the eye at different pressures ranging from 20 to 30 mm. Hg and found a linear relationship between inflow and pressure over this range.

In attempting to interpret the pressure/infusion curve derived in this study, use will again be made of the basic steady state hydrodynamic relationship. In this equation P_o is known and F, as stated previously in this paper, is believed to be eight to 12 µl. min^{-1}; P_v has not been measured in cats but there is no reason to believe that this will differ significantly from the value of 9.0 to 10 mm. Hg found in rabbit and in human eyes (Goldmann 1947; Kornblueth & Linner 1955). Therefore, on the assumption that P_v equals 10 mm. Hg. and that C remains constant, three theoretic relationships have been calculated using values of F equal to 8, 10 and 12 µl. min^{-1}. These are plotted in Figure 7 and it will be seen that the experimental curve differs from the theoretical curves in showing positive curvature; therefore, under these experimental conditions, one or more of the parameters F, C, or P_v varied with

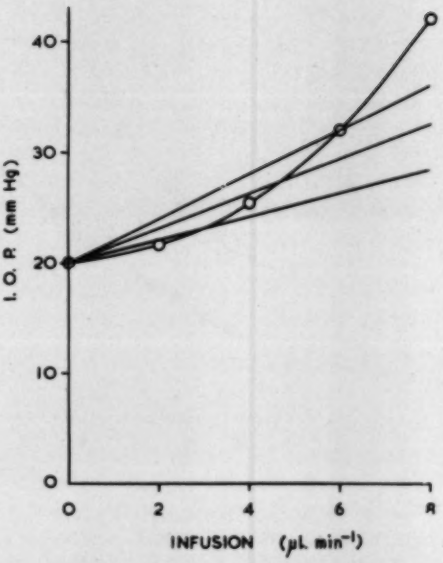

Fig. 7 (Langham). The influence of different rates of infusion on the steady state intraocular pressure. The three linear curves were calculated from the equation $P_o = F/C + P_v$ on the assumption that P_v and P_o were 9.0 and 20.3 mm. Hg. respectively and that the normal rate of flow of aqueous humour was 8, 10 or 12 µl min^{-1}. The mean pressure/infusion curve in the living cat is included for purposes of comparison (O———O).

the intraocular pressure. Evidence that an increase in pressure does decrease the rate of aqueous humour formation in cats has recently been found (Langham 1959a) and this could help explain the shallow slope of the pressure/infusion curve as the pressure approaches normal. On the other hand, the marked increase in slope of the curve at the higher infusion rates cannot be explained on this basis and it must be concluded that there was an increase in either the outflow resistance or P_v. In human eyes P_v has been found to vary little with large rises in the intraocular pressure and, therefore, it would appear more likely that the increased slope at pressures well above normal is due to a rise in the outflow resistance.

Summary

1. The relationship between the intraocular

pressure and different rates of infusion of fluid into the anterior chamber of cat eyes has been determined. In the living animal, the mean pressure/infusion relationship was found to have a positive curvature, while in the dead eye the steady state pressure increased approximately directly with the infusion rate over a pressure range of 10 to 35 mm. Hg.

2. In the course of these studies, manometric measurements were made of the intraocular pressure in cats anesthetized with Urethane. Values in the eyes of individual animals agreed closely and the mean pressure in 57 eyes of 36 adult cats was 20.1 ± 0.37 mm. Hg. After death the intraocular pressure fell rapidly to a mean value of 6.26 ± 0.56 mm. Hg. Any further decrease in pressure took place very slowly.

3. It is concluded that under these experimental conditions a change in intraocular pressure caused a compensatory modification of the intraocular dynamics that tended to minimize changes in the physiologic steady state pressure of the eye.

The Wilmer Institute, Johns Hopkins University Medical School (5).

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Discussion

Dr. Ludwig von Sallmann (Bethesda, Maryland): Dr. Langham has reported a fine piece of work conducted with the elegance of experimental technique and with the competent analysis of data which characterize his many investigations on aqueous humor dynamics. In a previous study, he has shown that in the freshly killed rabbit the steady-state intraocular pressure increased linearly with the rates of infusion into the anterior chamber. In contrast with this, in the living anesthetized animal, the pressure-infusion relationship was not linear, but when plotted, exhibited a positive curvature. This was thought to strengthen the concept that homeostatic reflexes are involved in modifying aqueous humor formation and outflow resistance. Since preganglionic sectioning of the sympathetic nerve abolished the typical responses of the living eye to different infusion rates, the hypothesis of a nervous mediation for the regulation of the intraocular pressure was considered.

Dr. Langham has now extended his comprehensive studies on the rabbit to the cat. The differences of the results in the two species were of quantitative and not qualitative nature; namely, the positive curvature, expressing the nonlinear pressure-infusion relationship, was also obtained in the living eye of the cat and essentially a straight-line function was demonstrated in the dead eye. Experimenting with the living cat eye, Dr. Macri of the National Institutes of Health previously described a curve which is comparable to that shown by Dr. Langham. Dr. Macri interpreted the shape of the curve as indicating a biphasic outflow pattern. He has also recorded, however, straight-line relation-

ships in some living cat eyes and has shown, at least once, in the human excised eye the positive curvature type. The significance of these exceptions from usual results is not clear at present. Dr. Langham might be so good to tell us whether he also has noted such uncommon patterns.

The suggestion that a homeostatic mechanism, mediated by the nervous system, affects intraocular pressure regulation does hold great attraction to workers of our laboratory where such nervous influences have been studied for a considerable number of years. There were in the last year, new developments which tend to dilute the evidence of a nervous regulatory mechanism. Lele and Grimes have observed that in the great majority of many experiments, increased afferent impulse activity of the posterior ciliary nerves caused by eye pressure increases was transient and that a sustained discharge rate in response to a constantly elevated eye pressure was a rare event in their series. This would indicate that the studied receptors are relatively rapidly adapting and therefore cannot serve well in long-term pressure regulations. The information available now is confined to short-term experiments and to the impulse rate in the large fibers of posterior ciliary nerves. These fibers represent only a small portion of the total population. Nothing can be said about the activity of the main bulk of small fibers so that the issue of afferent electric impulses and their connection with intraocular pressure changes is still not settled.

Dr. Langham assumes that the pressure in the episcleral veins in the cat might be of an order similar to that determined in the human and in the rabbit. Studies of Dr. Macri, now in press, deal with the relationship of the intraocular pressure and ocular venous pressure in the cat. He concluded that the two pressures differ less from each other than reported in other species and that there is a close interdependence of the changes of the two pressures. These findings do not alter, I believe, the conclusions based on Dr. Langham's calculations, but emphasized the need for distinguishing between species of laboratory animals when vascular and nervous responses are investigated.

From all this it is evident that our experience to date is insufficient to form a reliable opinion on the part homeostatic and nervous mechanisms play in the regulation of the intraocular pressure. The extension of studies as planned by Dr. Langham and also planned by our laboratory, is necessary for a more satisfactory insight in the physiology of intraocular pressure regulations.

DR. MAURICE E. LANGHAM (closing): I would like to thank Dr. von Sallmann for his very helpful comments.

The agreement between the conclusions drawn from this paper and the recent study of Macri is of interest in that Macri's conclusions were drawn from an analysis of pressure decay curves, i.e. nonsteady state phenomena.

I look forward to hearing Macri's latest observations on the variation of episcleral venous pressure with intra-ocular pressure in the cat's eye. However, as Dr. von Sallmann pointed out, changes in venous pressure would not modify the general conclusions drawn from this study.

THE OCULAR RIGIDITY FUNCTION*

MONTE G. HOLLAND, M.D., JAMES MADISON, M.D., AND WILLIAM BEAN, M.D.
New Orleans, Louisiana

PART I: EXPERIMENTAL TECHNIQUE

INTRODUCTION

By "ocular rigidity function" is meant a mathematical expression which relates pressure change to a corresponding volume change in the eye. This relation can be used to predict the pressure change which would result from a change in volume and conversely, by knowing a change in pressure it is possible to estimate the change in volume which should have occurred. It is thus a symbolic analogue used to represent certain pressure-volume events.

Because the ocular rigidity function is a means of making pressure-volume transformations, it is useful to both clinical ophthalmologists and investigators in ocular Physiology and Pharmacology. The calibration table of the clinical indentation tonometer is based on an estimate of the ocular rigidity function and similarly, in clinical and experimental tonography, it is used in calculations of the facility of outflow.

In the recent literature, there has been some difference of opinion concerning the

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nature of the formula which best approximates this function (Friedenwald,[1] McBain,[2] Macri,[3,4] Perkins,[5] and Prijot[6]). Because of the cardinal importance of the problem, the following investigation was conducted.

Since some of the disagreement may result from differences in experimental technique, we believe that a somewhat detailed description of methods is necessary.

A. Materials and Methods

Briefly, the experiments consisted of introducing small quantities of fluid (volume increments) into the anterior chamber of the enucleated eye of a cat and measuring the resultant pressure changes (pressure increments) at several constant initial pressures (P_i).

1. *Equipment.* The volume increments (ΔV) were delivered with a precision micrometer microburette (Micro-metric Instrument Co., Model SB-2) fitted with a one cc. tuberculin syringe.

A physiologic pressure transducer (Statham Instrument Co., Model P23BB, range zero to 5.0 cm Hg) was used to measure the pressure increments (ΔP) and the intraocular pressure.

The power supply for the pressure transducer in most experiments consisted of rectified direct current and in later experiments was a 12-volt automobile storage battery tapped to produce 10 volts. The latter proved to be superior in stability despite the use of a voltage regulating transformer with the former unit.

The recorder was a Leeds and Northrup potentiometric "Speedomax," with a range of zero to 10 mv.

2. *Equipment calibration and special precautions.* The microburette and syringe were carefully calibrated several times at room temperature with a 0.1 ml. pipette graduated in .001 ml. and the average number of scale divisions per microliter of fluid determined. The accuracy of the apparatus was considered to be satisfactory (1.0 μ l. = 2.163 scale divisions, with a standard deviation of .06553). It was linear over the range used. The plunger of the syringe was coated with high vacuum grease (Dow Corning) to prevent leakage. Special 1.0 cc. syringes (B & D, 1YT) of a more uniform bore were tried but were found unsatisfactory because of a tendency for the barrel to split when inserting the plunger after it had been coated with high vacuum grease.

The pressure transducer was calibrated prior to each experiment with a water manometer from zero to 50 mm. Hg, with the pressure diaphragm of the transducer, the needles for canulating the eye and the zero meniscus of the manometer all at the same level. This precaution was taken to eliminate any hydrostatic pressure gradient on the transducer or eye which would affect absolute pressure measurements. The absolute and differential pressures could be measured with an accuracy of approximately $\pm$ 1.0 percent. Special care was taken not to exceed the manufacturer's specifications on voltage and pressure overload (a maximum of 12 volts, or 50 mm. Hg). In early experiments pressure overloads were inadvertently sustained while cleaning the instrument, producing a loss in sensitivity and necessitating repairs. The loss in sensitivity highlighted the necessity of not exceeding the pressure limit of 50 mm. Hg. This was prevented in later experiments by monitoring the transducer with a recorder whenever it was being handled. When higher pressure ranges were studied, a transducer with a special diaphragm (Statham Instrument Co.) was used which could withstand the pressure overload without loss of sensitivity or linearity. It should be emphasized that to maintain accuracy in pressure measurements, the utmost care must be taken to avoid even momentary pressure overloads, either when cleaning the instrument or during experiments.

The pressure diaphragm of the transducer was checked separately for linearity by connecting the calibrated syringe of the microburette directly to the transducer and deter-

mining the volume displacements over the experimental pressure range. The transducer, syringe and recorder were linear over the range zero to 50 mm. Hg.

The equipment was then reassembled with connections between the transducer and microburette made with polyethylene tubing (Clay-Adams Co., PE 190, PE 50) used in the experiments. The volume displacement required to produce a given change in pressure in the "closed system" was determined and used as a correction for the volume increments in the experiments. The entire system was linear over the stated range.

When checking for leaks prior to the latter calibrations, it was observed that the pressure in the "closed system" fluctuated considerably because of extreme sensitivity to temperature changes. It was only possible to eliminate these fluctuations by immersing all of the fluid filled components in a constant temperature bath (37°C. ± .05°). Under these conditions, the system could be demonstrated to be free of any significant leaks. The pressure could be maintained at 40 to 50 mm. Hg for 30 minutes or longer, with a pressure loss never exceeding 0.25 to 0.5 mm. Hg. All subsequent calibrations and experiments were done with controlled temperature.

3. *Experimental procedure.* Cats of both sexes ranging from 1.5 to 5.0 kg. were used in all experiments. The animals were anesthetized with intraperitoneal Nembutal (35 mg./kg). Alternating right with left eyes in succeeding experiments, one eye was enucleated. The animal was kept alive until the conclusion of the experiment. After killing the animal with intracardiac Nembutal, the opposite eye was enucleated.

Each eye was carefully cleaned of fascia, blood and so forth, and the optic nerve trimmed close to the sclera. It was inspected for nicks or cuts and discarded if any were present. The eye was weighed on an analytic balance (1.0 mg. sensitivity) while kept moist. In previous experiments, with 18 eyes, the density of the globe had been estimated by Archimedes principle. The average density was 1.0415, with a standard deviation of 3.8×10^{-3}. After weighing, the volume of the globe was calculated.

The eye was transferred to a constant temperature bath containing a modified Krebs-Ringer's solution buffered to pH 7.4, the syringe of the microburette having previously been filled with this liquid. The globe was placed in the well of a platform-like holding device, resting on a gauze pledget. Two 25-gauge needles with short bevels were soldered together and connected (via PE 50) to two three-way stopcocks which had been sealed with high vacuum grease and tested for leaks separately. One stopcock was connected to the pressure transducer (by PE 190) and the other with the syringe of the microburette (also with PE 190). The entire system was immersed in the constant temperataure bath (fig. 1). Special precautions were taken to insure that the transducer, syringe of the microburette and the connecting tubing were free of air bubbles and leaks. With the eye submerged in the solution,* the double needle was inserted into the anterior chamber near the limbus. The pressure transducer was shunted out of the system by means of the stopcock while inserting the needles to avoid a pressure overload. Using the magnification of the Zeiss Otoscope, the needles were inspected in situ to determine whether they were in proper position. If any damage to the anterior segment had been sustained from the insertion of the needles, or if any small air bubbles were present, the eye was discarded. By inserting the needles with the eye immersed in the bath, the latter difficulty usually was prevented.

In early experiments a constant volume increment was delivered to the eye in a stepwise fashion, allowing a five to 10 second interval between infusions (fig. 2). This we designate as a "filling" or "loading" curve, and is similar to the technique followed by

* The cornea was immersed to a depth never exceeding 1.0 cm. below the level of the fluid.

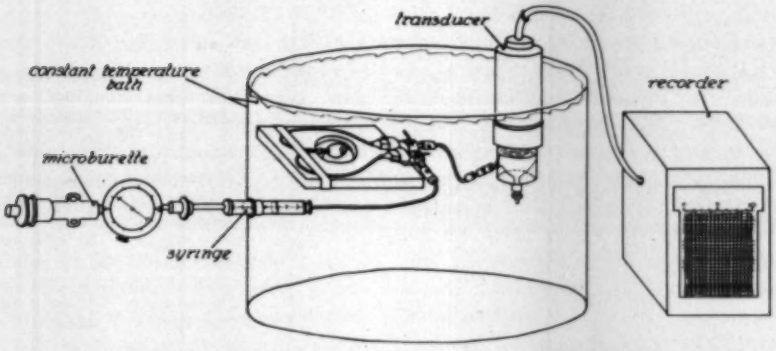

Fig. 1 (Holland, Madison and Bean). Schematic representation of the equipment showing fluid-filled components of the system immersed in a constant temperature bath.

Prijot,[6] Macri,[3] and McBain.[2] Because of certain technical limitations which will be discussed later, the experimental procedure was changed to the following which we designate as an "emptying" or "unloading" curve (fig. 3). The intraocular pressure was slowly elevated to slightly above the highest initial pressure (usually about 37.5 mm. Hg) and allowed to decay to the first initial pressure. At this point, approximately 2.3 microliters of fluid were delivered to the eye as rapidly as possible, yet consistent with accurate delivery. The time delay between the start of the infusion and the pressure maximum was usually of the order of 1.0 to 1.5 seconds. The pressure decayed spontaneously until the same initial pressure was reached, at which time another slightly larger volume increment was given. This procedure was followed until seven volume increments were given, each slightly larger than the preceding and all starting from the same initial pressure. Following this, the pressure was allowed to decay spontaneously to the next initial pressure, usually at 35 mm. Hg for the second series of seven volume increments. Seven initial pressures, ranging from 37.5 mm. to 10 mm. Hg, were investigated in this manner. The volume increments were constant for all experiments and ranged from 2.3 to 9.2 microliters. The actual volume entering the globe at the various initial pressures varied somewhat, depending on the volume absorbed by the measuring equipment and the rate of outflow from the eye.

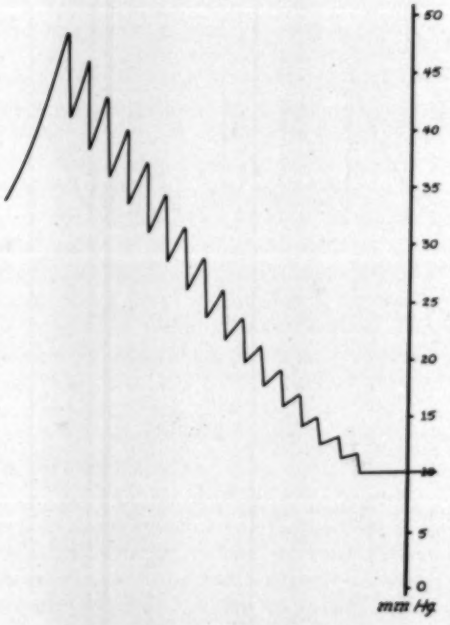

Fig. 2 (Holland, Madison and Bean). A "filling" or "loading" curve in which the intraocular pressure is elevated in a step-wise manner by a serially administered constant volume increment.

At the conclusion of each experiment the eye was removed from the constant tempera-

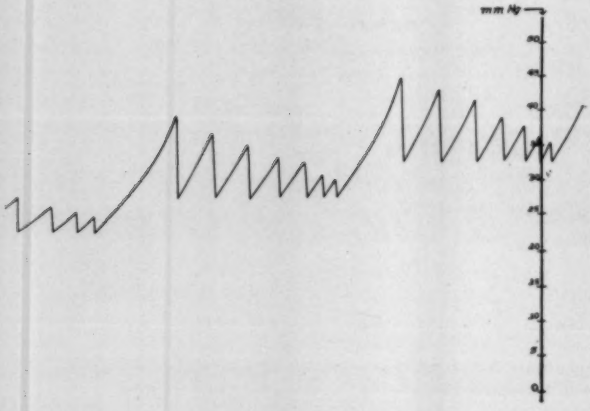

Fig. 3 (Holland, Madison and Bean). A "emptying" or "unloading" curve. The intraocular pressure is slowly elevated to the highest initial pressure. Seven volume increments are given sequentially, each slightly larger than the preceding and all starting from the same initial pressure. The intraocular pressure is allowed to fall spontaneously to the next lower initial pressure, where the series of volume increments is repeated.

ture bath. The intraocular pressure was maintained at 50 mm. Hg while the entrance point of the needles was inspected under magnification to determine if leaks were present. In earlier experiments, fluorescein was added to the perfusing fluid as a further safeguard against leakage. If evidence of leakage was observed by either method, the experiment was discarded. The experiments on the average lasted approximately 30 minutes.

4. *The experimental data.* To get the best possible approximation to the actual volume increments delivered to the eye, two important corrections must be made. The first has already been mentioned, that is, the volume error of the transducer and connecting tubing. The volume of fluid displaced in the tubing and the transducer for a given change in pressure was determined by the pressure-volume calibration of the "closed system." All volume increments were corrected for this factor.

A second source of volume increment error is the volume which leaves the eye by outflow during the time interval from the beginning of the infusion until the pressure maximum is reached. Macri[3] and co-workers have shown experimentally that by extrapolating along the pressure-decay curve to the time of the beginning of the infusion, the pressure increment can be corrected for the loss of fluid by outflow (fig. 4).

The following observation lends support to this procedure. Under the same experimental conditions, usually there is little difference in the pressure increments observed

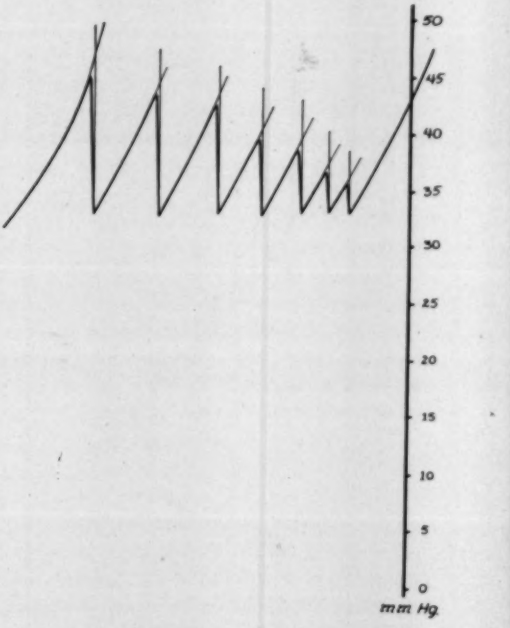

Fig. 4 (Holland, Madison and Bean). By extrapolating back along the outflow curve to the beginning of the infusion, the pressure increments can be corrected for the fluid lost by outflow.

in the eyes of the same animal. If the rate of outflow is different in the two eyes, the pressure increments under the same conditions may be significantly different. This difference becomes small, however, when the pressure increments are corrected by extrapolation.

The logic of the method is to estimate the pressure increment which would have resulted if there had been no fluid lost by outflow from the eye. This correction is probably slightly underestimated. Extrapolating along a tangent, to a curve which is convex toward the abscissa, will yield a point which is below the curve, in proportion to the distance extrapolated and the rate of change in the tangent. Since the interval over which the extrapolation is carried out is small (1.0 to 1.5 seconds), the error was considered to be not significant.

The data for a single experiment consisted of seven corrected volume increments and seven extrapolated pressure increments for each of seven constant initial pressures. In this investigation, over 250 experiments were performed until the technique was developed as fully as possible. A series of 50 experiments with initial pressures ranging from 37.5 to 10 mm. Hg and a series of 10, with P_I ranging from 70 to 15 mm. Hg*, were selected for numerical analysis (see Part II). These experiments were selected because they were considered to be as free as possible from technical imperfections. The number of individual points on pressure-volume graphs subjected to curve approximation analysis was over 3,000.

B. Discussion

1. *Temperature control.* Experimentally in the "closed system" with the microburette connected directly to the pressure transducer, it was impossible to achieve a stable pressure indication at a given volume without an isothermal environment.

It is possible to calculate the volume expansion of a given quantity of fluid in such a system which would result from a 0.5°C ambient temperature change. If this change in temperature occurred while delivering the smallest volume increment used in the experiment (2.3 microliters), approximately a 50 percent error in the estimated volume increment could result. By placing the fluid filled components of the system in an isothermal environment this error would be minimized.

From a thermodynamic point of view it is known that the elastic properties of a material will vary with the temperature.[8]

In view of these considerations, we believe that it is absolutely necessary to maintain the entire apparatus, as far as possible, in an isothermal environment† when measuring the ocular rigidity of the enucleated eye.‡

2. *The external environment of the eye.* If an isothermal environment is necessary, it is logical to question whether the fluid bathing the globe has any influence on the measurements. In 35 early experiments, ordinary tap water was used in the constant temperature bath. In the next 15 experiments, the fluid was changed to unbuffered normal saline. The experimental data from these two groups were analyzed by curve approximation methods using the IBM 650 Digital Computer.[9] The procedure used was as follows: at each of seven distinct and constant initial pressures, the experimental data ($Y = \Delta P$, $X = \Delta V$) were tested to determine which of three elementary functions best approximated the data. The functions tested were: linear, $Y = aX + b$; exponential, $Y = be^{aX}$ and a power curve through the origin, $Y = bX^a$. The method of least squares was used with the standard error of estimate, $S_{y.x}$ as a measure of approxima-

* A special tranducer was used for this range to avoid damaging pressure overload.

† Prijot[4] and Ridley[7] have attempted to control the temperature in their experiments.

‡ A detailed investigation of the effect of temperature changes on ocular rigidity is in preparation for publication.

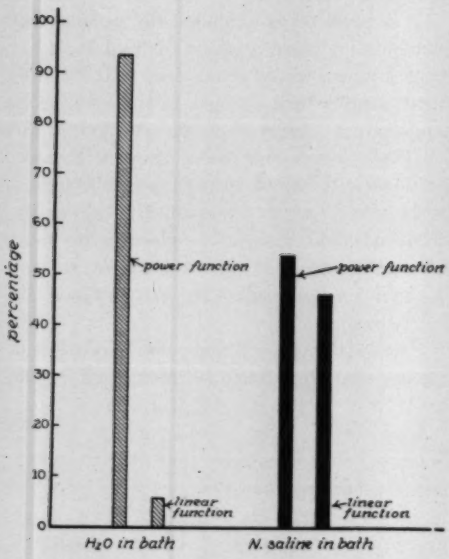

Fig. 5 (Holland, Madison and Bean). Different fluids in the constant temperature bath have apparently influenced the best approximating function for the experimental data.

tion. The standard error was summed for each curve at each initial pressure and the sum used as a criterion for best approximation over the range of initial pressures. The results were somewhat surprising (fig. 5).

With tap water in the constant temperature bath, 94 percent of the experiments were approximated best by the power function; in the series with unbuffered saline in the bath, 53 percent were best approximated with a power function, and 47 percent with a linear function. The perfusing fluid was also varied (unbuffered normal saline, normal saline buffered to pH 7.4, balanced salt solution of Alcon Co., and Ringer's) during these experiments but differences in the best approximating curve had no apparent correlation with this factor. It was concluded that the different fluids in the constant temperature bath probably were responsible for the observed differences, since the experiments were otherwise identical in every detail.

On the basis of this evidence, it was decided to control the external and internal environments of the globe by using a modified Krebs-Ringer's solution buffered to pH 7.4 in the constant temperature bath as well as for the perfusing fluid. These further restrictions would seem to preserve the globe in a more nearly physiologic milieu. All subsequent experiments were done under these conditions and constitute the ones subjected to numerical analysis.

3. *"Filling" versus "emptying" experiments.* The fact that the eye will not only respond elastically to a deformation (tonometric or infusional) but will also stretch under stress (creep, or stress relaxation), has been reported in the literature (Ridley,[7] Macri,[4] Goldmann[11]). It is also known that an eye subjected to high pressures, as in glaucoma, may manifest an increased rigidity (Friedenwald[1]), which is interpreted to mean that it is in a stretched condition. In early experiments of the "filling" or "loading" type, it was observed that if another volume increment was given at the same initial pressure as one of the preceding increments, a higher ΔP resulted. If it were repeated, the third ΔP was again slightly higher. It then appeared to stabilize (fig. 6). A similar phenomenon was noted by Gloster, Perkins and Pommier.[10]

Because the most likely explanation of this phenomenon is creep, stretch, or viscous deformation, the experiments of the "emptying" or "unloading" type were designed to try to minimize this effect, the rationale being that by slowly bringing the eye to the highest desired initial pressure, some time will have been allowed for the eye to reach equilibrium in stretching. There is evidence that the stretch occurs rapidly (Gloster[10]).

Stretch should be minimized by the "emptying" or "unloading" experiments in contrast to the "filling" or "loading" experiments, since creep or stretch can be theoretically minimized by diminishing the stress; in other words, the closest approximation to a constant deformation in a visco-elastic material (such as the eye), is obtained when the

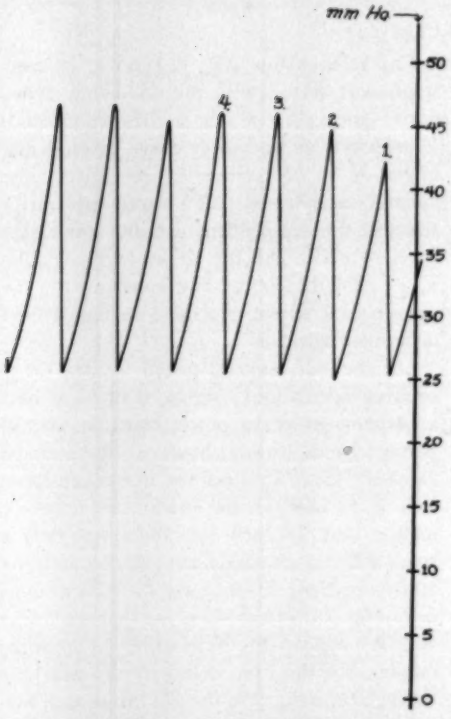

Fig. 6 (Holland, Madison and Bean). Serial repetition of a constant volume increment starting from a constant initial pressure results in slightly larger pressure increments for approximately three infusions.

stress is diminished or relaxed (Reiner[12]).

Many other considerations made it necessary to perform the experiments in this manner, not the least important being that more information was obtained because the volume increments could be varied independently. The analysis of data from experiments of this type has provided new information concerning the visco-elastic properties of the eye, not apparent with older methods (see Part II).

4. *Summary and Conclusions.* We have described our experimental technique for the study of ocular rigidity in the enucleated cat eye. In the experiments, special precautions were taken to insure that: (1) there was minimal trauma to the eye; (2) the equipment was accurately calibrated; (3) volume increment errors due to the displacement in the measuring equipment and due to outflow were corrected; (4) there were no leaks in the assembled equipment; (5) there was no air in the system; (6) there was constant temperature control; (7) the pH and ionic composition of fluids bathing the eye and infused into the eye were maintained in a range which would seem to preserve the globe in an environment more nearly approximating the physiologic state; (8) there were no leaks around the needles at the end of an experiment; (9) stretch or creep was minimized by limiting the range of pressures in most experiments to 50 mm. Hg, and by performing "unloading" or "emptying" experiments.

Part II: Curve approximation analysis

Introduction

In a recent study of the human eye, McBain[2] has shown that the widely used formula of Friedenwald[1] is not the best approximation of the ocular rigidity function. Several investigators (McBain,[2] Macri,[3,4] Perkins,[5] Prijot[6]) have observed that the Friedenwald coefficient of scleral rigidity, k, is not constant but depends on the pressure at which it is measured. In most animal species studied k was found to vary inversely with the pressure.*

A unique solution to the problem of finding a function which completely characterizes the visco-elastic properties of the eye can never be attained by empirical curve approximation analysis, since there is an infinite number of functions which could be tested. The best result attainable from an analysis of this type would be a function which approximated the data to such a degree, that from pragmatic considerations further refinement would appear unnecessary. However, as pragmatic considerations change, the conception of a satisfactory ap-

* In unpublished experiments the authors have confirmed this result.

proximation would also be subject to change. Clearly, a definitive solution of this problem can only be derived from a thorough and rigorous theoretical analysis.

It is the purpose of this investigation to report the results of empirical curve approximation analysis of the experimental data presented in Part I of this study. A possible basis for a theoretical characterization of the visco-elastic properties of the eye is then indicated.

Methods

The data from each experiment consisted of a set of seven points (ΔP, ΔV) for each of seven constant initial pressures (P_i). Because of the large number of experimental points subjected to analysis, it was necessary to employ a digital computer for the computations.[9] In 1.5 minutes the IBM 650 Digital Computer electronically processed the same data which required three man days with a desk calculating machine. At this rate, in excess of ten man years would have been required to manually process all the data analysed in this report. The computer had the added advantage of being less likely to make errors in computation.

All curve approximations were programmed for the method of least squares using the standard error of estimate, $S_{y.x}$, as a measure of approximation. The least squares calculations require proper choice of dependent and independent variables.[13] For each function tested, the reasons for selecting the dependent variable will be specified.

If the experimental points do not appear to fall on a curve passing through the origin, or if there is no theoretical reason for supposing that they should, provision must be made for this extra degree of freedom when selecting a function to be tested—otherwise spurious results may be obtained.

The results of the analysis will be divided into three parts corresponding to the problems considered and the computer programs designed to deal with them.

Results

Part I.

The relationship, $\Delta P = f(\Delta V)$, P_i constant, was tested with the following functions: linear, $Y = aX + b$; exponential, $Y = b\,e^{aX}$; and a power curve through the origin, $Y = bX^a$. Since experimentally the volume increments (ΔV) were arbitrarily selected, the independent variable was clearly $X = \Delta V$ and the dependent variable, $Y = \Delta P$; that is, at any constant P_i, the pressure increment depended on the volume increment selected.

Of the 427 curves tested (1,281 least squares calculations), 62 percent were best approximated by the power function, and 38 percent by the linear; however, the average "scatter," $S_{y.x}$, was less for the linear function. In no case was the exponential approximation best. In each case there was only a small difference between the power and linear approximations. In studying the data graphically (fig. 7), it was observed that the linear function appeared to be an excellent approximation, but the lines definitely did not pass through the origin. In the 427 linear approximations, 420 had negative y-intercepts. The exponents of the power approximations were always greater than, but close to 1.0 (average 1.116). It was also observed that large exponents occurred whenever the y-intercept was large (more negative).

Since the power function used for the approximation constrained the curve to pass through the origin, it appeared likely that an exponent >1 was a spurious result of this restriction. To remove this restriction, the translation $Y = \Delta P + c$ was performed where c represents the y-intercept of the linear approximations. The effect of this translation was to move the curves through or very close to the origin without changing the type of function represented by the data, or the slopes of the linear approximations (fig. 8).

The data were then reprocessed by the same program with the result that 426 out

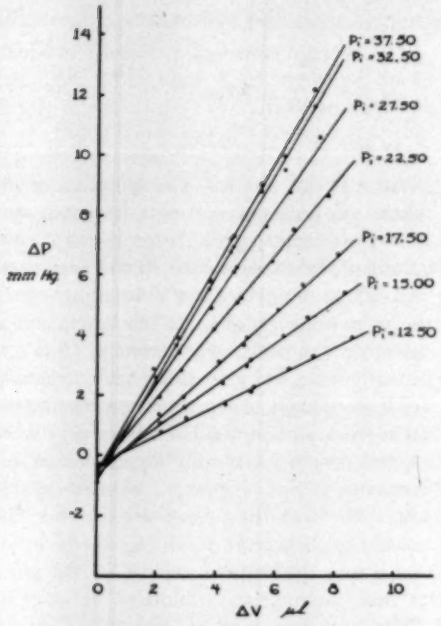

Fig. 7 (Holland, Madison and Bean). ΔP as a function of ΔV at constant initial pressures (P_i). The slopes and intercepts vary with P_i. The general equation of these lines is $\Delta P = E(P)\Delta V - Ce(P)$.

of 427 curves were best approximated by a straight line through the origin.*

Thus, of the three functions tested without legitimate exception, the data were best approximated by the function:

$$Y = EX \qquad (1)$$

where $Y = \Delta P + C_e$ and $X = \Delta V$.

On substituting, the following equations result:

$$\Delta P + C_e = E\Delta V \qquad (2)$$

$$\Delta P = E\Delta V - C_e \qquad P_i \text{ constant} \qquad (3)$$

Inspection of Figure 7 shows that both E and C_e vary with the initial pressure. In the next section the relation of E to the initial pressure was investigated.

PART II. $E = g(P_i)$

Since the slope, E, of Equation (3) varied with the initial pressure, the relation, $E = g(P_i)$, was tested by making the transformation $Y = E$, $X = P$, and performing the same curve approximations as in Part I. The initial pressure was the independent variable since it was arbitrarily selected and E depended on the initial pressure at which it was measured.

The approximations were poor because the data did not tend to fall on a smooth curve passing through the origin. The curve was also evidently nonlinear (fig. 9).

To approximate this relationship, two functions were selected which empirically resembled the experimental curve, namely, an

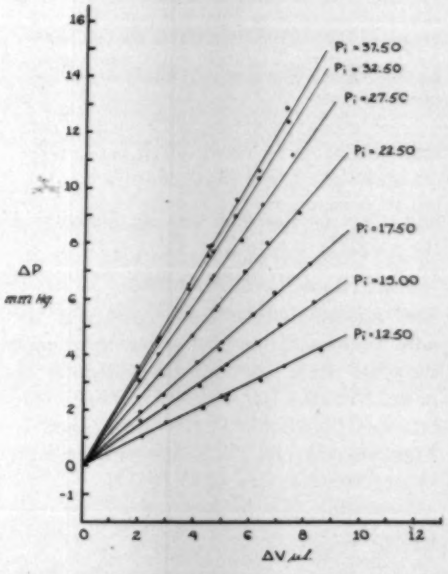

Fig. 8 (Holland, Madison and Bean). The stress (ΔP) data of Figure 7 have been corrected by the translation of $\Delta P = (\Delta P + Ce)$ to bring the lines through the origin. The general equation of these lines is $\Delta P = E(P)\ \Delta V$.

* In the one exception in which a power approximation was best, the difference in $S_{y \cdot x}$ of the power and linear approximations occurred in the sixth decimal place. The power exponent was .99901.

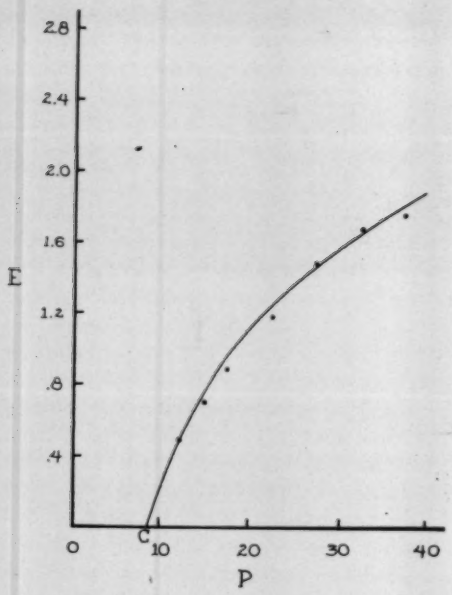

Fig. 9 (Holland, Madison and Bean). E, the slopes of the lines in Figure 8, as a function of pressure. The solid line represents the best approximating function, $E = b\ (P - c)^a$, where c is the intercept on the pressure axis. The dots are experimental points.

exponential curve rising to an asymptote,

$$Y - k = be^{aX} \qquad (4)$$

and a power function with an intercept on the x-axis,

$$Y = b(X - c)^a.* \qquad (5)$$

The approximations were good and with only three exceptions out of 60 experiments, the data were best approximated by the power function (equation 5). The average $S_{y.x}$ for Equation (4) was .0800, and for Equation (5) .0512. The standard deviation in the latter case was .0252.

Rewriting (5) in pressure-volume notation gives:

$$E = b(P - c)^a \qquad (5a)$$

The average values of a, b and c obtained from Equation (5a) were .389, .589 and 10.71, respectively.

PART III.

An attempt was made to graphically integrate the experimental data by using tangent lines constructed from the ΔP, ΔV values at the experimental initial pressures.

In integrating from low to high pressures, the volume of the globe at the lowest initial pressure (an unknown quantity) was arbitrarily assigned the value zero. Starting from this lowest P_1, the pressure increments (corrected for the negative intercepts) were plotted versus the serially larger volume increments. A line tangent to the curve at this lowest P_1 was thus constructed. This line usually included the next higher P_1 in its range and the relative volume of the globe at this pressure was estimated graphically. This procedure was carried out over the range of experimental P_1, and a smooth curve drawn connecting these points.

The same data were graphically integrated starting from the highest P_1 and proceeding to the lowest. At the highest P_1, the pressure increments were plotted versus the volume increments to determine the tangent line at this pressure. By extrapolating back along the tangent to the next lower P_1, the relative volume at this latter pressure could be estimated graphically. The procedure was repeated for each initial pressure until the lowest was reached. The relative volume of the globe at this pressure was arbitrarily given the value zero.

The results of the graphical integrations are compared with the analytically integrated curve (see discussion) in Figure 10. Curve I is the result of graphically integrating from low to high pressures; curve II, from high to low; and curve III, the analytically integrated curve. The differences between curve I and curve II are attributable to inaccuracies inherent in the graphical integration method. If curves I and II are averaged,

* The details of approximating with these formulas are omitted. In general, the procedure followed was a least squares solution with an arbitrary constant (graphically estimated), combined with an iterative evaluation of the standard error of estimate as a function of the constants c or k.

the result agrees closely with the analytically integrated curve (fig. 11).

Discussion

1. *Interpretation of the negative intercept, C_e, in Equation (3).* If the unextrapolated pressure increments (that is ΔP uncorrected for the volume lost by outflow) are plotted versus the corresponding volume increments at a constant P_I, it is observed that the line falls far below the origin (line A, fig. 12). If the pressure increments are corrected for the fluid lost by outflow (that is, by extrapolation), the line is moved closer to the origin (line B, fig. 12). However, *after this correction the lines still fall below the origin*, that is, they have negative intercepts on the ΔP-axis.

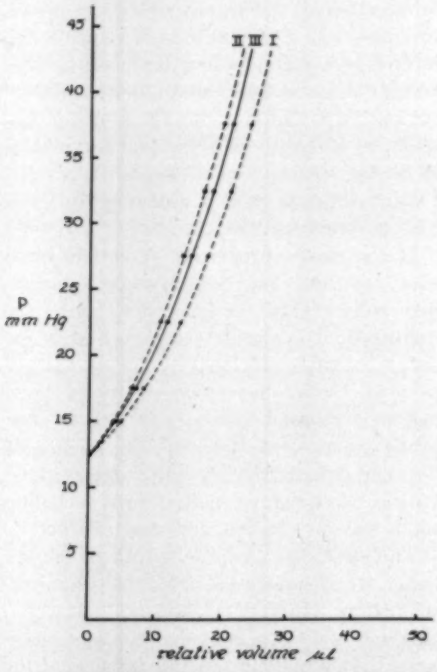

Fig. 10 (Holland, Madison and Bean). Curve I shows the result of graphic integration of the data from low to high pressures; Curve II graphic integration from high to low pressure; Curve III is the analytically integrated function shown in Figure 9.

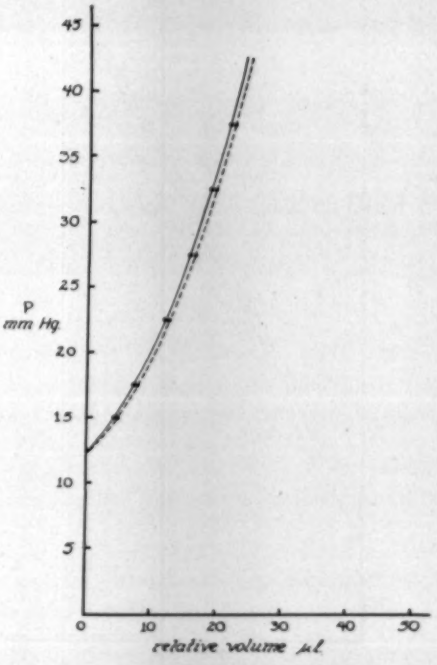

Fig. 11 (Holland, Madison and Bean). Dotted line average of curves I and II of Figure 10. Solid line represents analytically integrated curve.

The magnitude of the intercept had no relation to the degree of approximation of the linear function.

It would seem that either the correction of stress (ΔP) errors due to outflow by extrapolation produced a consistent underestimation, or stress relaxation occurred, or both.

The experimental records were inspected to determine whether or not errors in extrapolation could possibly account for the negative intercepts. Whenever the intercepts were small ($C_e < 0.4$ mm. Hg), this source of error seemed to be a plausible explanation. However, intercepts greater than this were too large to be explained convincingly on this basis.

It therefore appeared that creep or stress relaxation was also involved. Examined from this point of view, line B in Figure 12 would indicate that when $\Delta P = 0$, there had

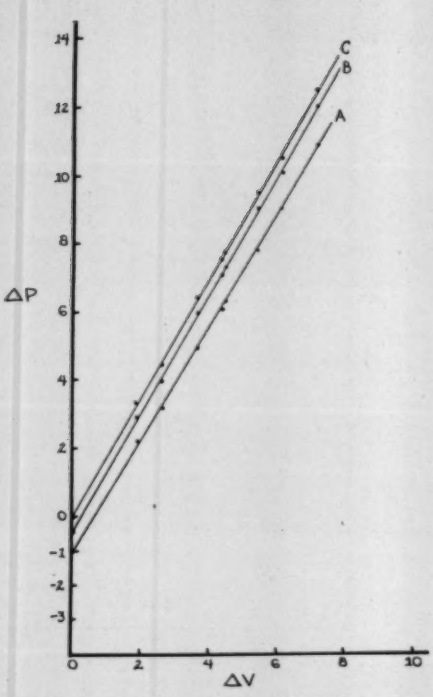

Fig. 12 (Holland, Madison and Bean). Line A, ΔP uncorrected for outflow errors. Line B, ΔP corrected for outflow errors by extrapolation. Line C, ΔP corrected to bring line B through the origin.

been some positive change in volume (stretch); or conversely, when $\Delta V = 0$, there had been a relaxation of stress $(-\Delta P)$.

Regardless of the explanation of the negative intercepts, it is clear that in order to characterize the distensibility of the eye unaffected by creep (or possible error), that the linear approximations should be corrected to pass through the origin. It is also evident that the correction should be applied to the stress (ΔP). Henceforward, the latter symbol should be interpreted as the stress thus corrected, unless it is otherwise defined in context.

2. *Analytical integration of* $E = g(P)$. Equation (1) then represents the relation of the corrected stress to the volume increments at a constant P_i. Rewriting Equation (1) and taking into consideration E as a function of pressure, the relation $\Delta P/\Delta V = E(P)$ is obtained. Using Equation (5a) and separating variables, yields:

$$\Delta V = \frac{\Delta P}{b(P-c)^a}, \tag{6}$$

which when integrated gives equation 7.

$$V = \frac{(P-c)^{1-a}}{b(1-a)} + D. \tag{7}$$

The constant of integration was evaluated by setting $V = 0$ at the lowest initial pressure. The analytically integrated function and an average graphically integrated curve are compared in Figure 11.

3. *Theoretical considerations.* Under the action of forces, a body may be deformed in several ways. The deformation is classified as elastic if it disappears after the forces are removed; it is plastic if it remains deformed permanently when the forces are removed; and it is considered a viscous deformation (the material flows) if under the action of forces the deformation continually increases without limit (Reiner,[12] p. 19). Plastic deformation is a special type of viscous deformation (Reiner[14]).

If the elastic properties of certain complex materials (as for example: plastics, high polymers, tar, dough and so forth) are measured, it is easily determined that the simple equation of Hooke's law is not applicable. Under the action of forces these materials respond viscously as well as elastically. In modern rheology, the science of flow and deformation of matter, the point of view is taken that all matter flows, including solids such as concrete and steel (Reiner[14]).

The effects of creep or stretch in the eye under stress have been noted by several investigators (Ridley,[7] Goldmann,[11] Macri[4]), although in the eye, creep has not been quantitated. This notwithstanding, because of the theoretical considerations mentioned in this paper, it should not be surprising that the eye is a visco-elastic material.

One rheologic method of dealing with

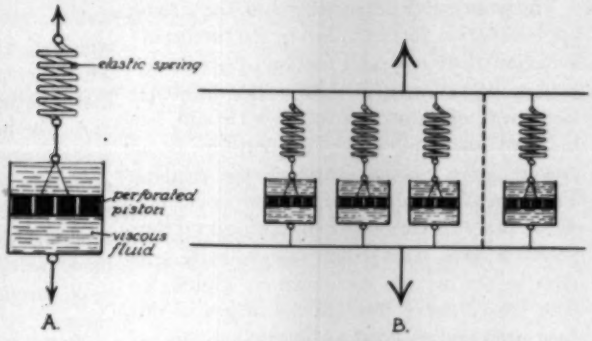

Fig. 13 (Holland, Madison and Bean). (Part A) The Maxwell-body, a series combination of a Hooke-solid (spring) with a Newtonian liquid (dashpot). (Part B) Generalized Maxwell Model.

these complex materials is to construct a theoretical model made up of the fundamental rheologic bodies (or combinations of them) which manifest the same type of visco-elastic properties as the material being studied. Perhaps the simplest model showing visco-elastic behavior is the Maxwell-body (fig. 13A), a combination of a Hooke—solid (spring) in series with a Newtonian liquid (dashpot). The Maxwell-body responds to a force with an instantaneous elastic elongation together with a steady Newtonian flow (Green[13]). The equation for this rheologic system is (Mark and Tobolsky,[8] p. 331):

$$ds/dt = (1/E)(df/dt) - (1/\tau E)f \qquad (8)$$

where s is a component of strain, f is a component of stress, E the elastic modulus, and τ is the relaxation time of the system.

Solving for df:

$$df = Eds - (1/\tau)Pdt, \qquad (9)$$

and using pressure-volume notation gives,

$$\Delta P = E\Delta V - (1/\tau)Pdt \qquad (10)$$

Equation (10) is very similar to Equation (3) of Part I, namely:

$$\Delta P = E\Delta V - C_e.$$

The equation of visco-elastic deformation of the Maxwell-body is analogous to that of the eye at a constant initial pressure. From this analogy, $-C_e$, would be interpreted as the relaxation of stress due to viscous deformation. However, a single Maxwell element is not sufficient for all initial pressures because E, the elastic modulus, varies continuously as a function of pressure. The generalized Maxwell case (fig. 13B) would seem required for a more complete description of the system. Prior to seriously considering this latter problem, however, several important and difficult questions would have to be answered.

4. *Previous results in the literature.* If the differential form of the Friedenwald[1] approximation formula is examined, the relation between $\Delta P/\Delta V$ and P is:

$$\Delta P/\Delta V = kP \qquad (11)$$

where k is the coefficient of scleral rigidity. The formula indicates that a plot of $\Delta P/\Delta V$ versus P would result in a straight line through the origin with slope k. An examination of Figure 9 shows that this is not the case, and in fact, a power function not through the origin was the best approximation of all the functions tested. In no case was Equation (11) the best approximating function.

McBain[2] has proposed the following empirical approximation formula:

$$V = aP^b + C. \qquad (12)$$

The approximation formula which resulted from the present investigation is very similar, namely:

$$V = \frac{(P-c)^{1-a}}{b(1-a)} + D. \qquad (7)$$

The principal difference between these two approximation formulas lies in the predicted behavior of dP/dV as a fuction of pressure, that is, $dP/dV = g(P)$. From the differential form of Equation (12) it would be inferred that the latter relationship is a power curve passing through the origin. Equation (7) in differential form would indicate that this curve had an intercept on the pressure axis. This latter case actually occurs in the cat eye as shown in Figure 9. The power curve through the origin in no case produced as good an approximation as a power curve with an intercept on the pressure axis. The intercept would indicate that the intraocular pressure must be brought to this level before the walls of the globe are elastically stressed. By way of analogy, in a system consisting of a partially collapsed rubber balloon, the volume must first be filled before its walls are subjected to the stress of elastic deformation.

Gloster, Perkins and Pommier,[10] in a study of the extensibility of excised strips of sclera, showed that on loading the strip there was an immediate elongation followed by a slow elongation. The first effect was evidently elastic and the second a viscous deformation, or creep. These results agree very well with the theoretical behavior of the Maxwell-body.*

Summary and Conclusions

The experimental data from Part I of this investigation were subjected to empirical curve approximation analysis using the IBM 650 Digital Computer. Several elementary functions were tested to determine which best approximated the data. The results of the analysis are summarized in the following conclusions.

1. The relation of pressure increments, ΔP, to corresponding volume increments, ΔV, given at a constant initial pressure, was best approximated by the linear function:

$$\Delta P = E\Delta V - C_e,$$

where E, the slope of the line and C_e, the ΔP intercept, varied as functions of the initial pressure.

2. The negative intercept of this equation was considered to be the result of an underestimation of stress (ΔP) corrected for errors due to outflow and stress relaxation (creep). When the stress was corrected for these factors, that is, the curves corrected to pass through the origin, the equation became:

$$\Delta P = E(P)\Delta V.$$

3. The slopes, E, as a function of pressure, were best approximated by a power function not passing through the origin, of the form:

$$E = b(P-c)^a.$$

4. Integration of this latter equation resulted in the formula:

$$V = \frac{(P-c)^{1-a}}{b(1-a)} + D$$

5. At a constant initial pressure, the visco-elastic properties of the eye are closely simulated by a rheologic model, the Maxwell-body. A generalized Maxwell model would appear necessary, although perhaps not sufficient, for a more complete characterization of the visco-elastic properties of the eye.

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Acknowledgments

The authors gratefully acknowledge the suggestions and helpful criticism of Dr. Lila Elveback, Professor of Biostatistics, Tulane University School of Medicine, and Mr. S. A. Orrell, Louisiana State University Medical School, Department of Pharmacology, for technologic advice and constructive criticism. Thanks are also due to Dr. Fred Schueler, Professor of Pharmacology, and Dr. James H. Allen, Professor of Ophthalmology, Tulane University School of Medicine, for their advice, support and stimulation. The authors are also indebted to Mr. William Nettleton, Tulane University Computer Center, for advice on computer programming problems and to Mr. Joseph Bellestri, Mr. Bruce Tischler and Mrs. Lorelle Deichmann for technical assistance in the project.

* Compare with Reiner,[12] p. 96 (Figure Viii, 2, $c > c_e$).

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Discussion

Dr. Elmer J. Ballintine (Cleveland, Ohio): This work is outstanding among investigations of scleral rigidity in meticulous attention to experimental details and in exhaustive statistical analysis of the many data. It will probably long remain our most reliable source of information on the scleral rigidity of the enucleated cat eye.

Apparently under the conditions of the experiments, the scleral rigidity is more sensitive to changes in the composition of the bathing solution than to changes in the perfusing solution. Is this effect of the external fluid the result of osmotic forces altering the state of hydration of the ocular coats?

I must protest one minor point in connection with the correction of the data for "run off" during addition of measured volumes of perfusate to the eye. The method of correction used by the authors results in an "overcorrection" of ΔP, not an undercorrection. As a simple calculation shows, the authors' method of extrapolation implicitly assumes that the injection occurred instantaneously, rather than over the finite period when the pressure was rising. The amount of plastic relaxation of the sclera must be, therefore, even more than calculated by the authors.

In Figure 7, which shows the family of straight lines relating ΔP to ΔV, there is an inherent contradiction in that if these are really straight lines, they should all have the same slope. For example, at the point on the line $P_1 = 12.50$ corresponding to $\Delta P = 2.5$ mm. Hg, the pressure within the eye is 15 mm. Hg. The curve beyond this point should be exactly the same as the curve marked $P_1 = 15.00$.

Inspection shows that at least five of the curves are slightly concave upward (that is, the end points lie on or above the line and the intermediate points on or below the line). These inconsistencies suggest that some function other than the ones investigated, might fit the data better.

Inspection of Figure 9 suggests that an excellent fit might be obtained by two straight lines; one through the first five points and the second through the last two points. Their intersection at a point corresponding to about $P = 30$ mm. Hg would indicate a discontinuity in the extensibility of the cat eye such as has been reported by others at this pressure.

It should be emphasized that the results obtained required a specific procedure and that the preliminary experiments were used as guides in the selection of conditions that would yield the most nearly reproducible results. It would be helpful in planning other investigations if, from their extensive experience, the authors could give us some estimate of the variability one might encounter among individual animals.

Would the authors speculate on how their results apply to the living cat eye? Is there any evidence to indicate that a similar pressure volume relation-

ship exists in the eye of the intact cat?

DR. MONTE G. HOLLAND (closing): I should like to thank Dr. Ballintine for his very interesting discussion. In the few minutes available to me I would like to answer some of the questions he has raised. I am sure Dr. Ballintine and I will have to spend some time privately on some of the points he has raised, because I cannot agree with him.

I am not prepared to give any reason for the sensitivity of the eye to the different fluids in which it was resting. A priori reasoning on biologic grounds would indicate that approximating a more nearly physiologic environment is desirable and that depriving a tissue of it might cause alterations in very sensitive parameters. I will talk to Dr. Ballintine later about whether the extrapolation produced an undercorrection or overcorrection. I don't think I should comment on that just now in the interest of economy of time.

There is no inconsistency in the curves in Fig. 4. In private conversations with him I mentioned this before. These slopes are linear approximations. The paper, when read carefully, states only that the best approximating function out of those tested was the linear one and that is all that is said.

Furthermore, the alleged inconsistency does not exist because these slopes are linear approximations of tangent lines at a point on the curve. So, if we extrapolate out along a tangent line we would expect some apparent discrepancy of the type Dr. Ballintine has pointed out.

We agree again that these lines might possibly be approximated by some other function, as Dr. Ballintine has suggested. That is precisely why we undertook this study. We did not want to guess which function might approximate it best. We wanted to measure it, and we did. We tried two straight lines, indicated in the last figure shown. We fitted the first part of the curve and then the entire curve, and we found that two straight lines did not give as good an approximation as the power function.

We did not select our techniques just on the basis of reproducible results, although that is obviously a desirable aim. In the first experiments which were done, the eyes were immersed in a constant temperature bath consisting of tap water. We had very consistent results. In thirty-five experiments the power function was the best approximation in every case, with only two exceptions. However, we decided we should investigate the possible influence of the environmental fluid, so we improved it and found that it made some difference. Because of this we did not continue to use tap water because we believe that there are sound reasons for trying to improve the environment of the globe. We have data on the scatter of individual eyes, and in the paper representative figures are given for each type of curve.

Again we hesitate to speculate about the application of this to in vivo eyes. We only wish to point out that much of our exact knowledge of the ocular rigidity function has been obtained from studies on enucleated eyes, and we realize of course that there are limitations to this technical approach.

I should like to thank Dr. Ballintine again, and thank you for your attention.

THE EFFECT OF OXYGEN TENSION ON GLUCOSE UPTAKE BY THE ISOLATED RABBIT LENS*

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AND
JOHN E. HARRIS, M.D.
Minneapolis, Minnesota

While exploring the factors governing cation balance in the lens, Dr. Harris observed that changes in lenticular cation content constituted a useful index of lens function in general. This observation led to the development of an in vitro technique for studying lens metabolism termed "the reversible cation shift."[1] Briefly, this procedure consists of causing an excised lens bathed in a nutrient medium to lose potassium and gain sodium by chilling at 0°C, then reversing the process by incubating at 37°C. The cold-induced cation shift can be reversed, however, only if the surrounding medium is appropriately constituted. Examination of the effects of altering the medium in various

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School (Portland) and the Department of Ophthalmology, University of Minnesota School of Medicine (Minneapolis). Supported by Grant No. B-1990 National Institute of Neurological Diseases and Blindness National Institutes of Health U.S. Public Health Service.

ways has yielded considerable information regarding the maintenance of normal cation contents by the lens. For example, lenticular cation balance is metabolically mediated and glucose appears to be the metabolite of choice for supporting reversal of the cold-induced cation shift as well as for supporting other energy-requiring processes in the lens.

The original experiments demonstrating that chilled lenses lose potassium and gain sodium employed Tyrode's solution as the nutrient medium.[2] Attempts to reverse these changes in cation content were unsuccessful until the potassium and bicarbonate ion concentrations of the bathing medium were increased to approximate those found in the aqueous humor.[3] Like the aqueous humor, this medium depends primarily upon the bicarbonate: carbonic acid buffer system for maintaining the pH at physiologic levels. Since the carbonic acid concentration of such a buffer system is a function of the partial pressure of carbon dioxide (pCO_2) in the overlying atmosphere, increasing the bicarbonate concentration of the medium required a corresponding increase in the pCO_2 of the gas phase if the ratio HCO_3^- : H_2CO_3 and hence the pH, of the solution were to be held constant. A bicarbonate ion concentration approximating that of the aqueous humor requires that the pCO_2 lie in the neighborhood of 40 to 45 mm Hg (five to six volumes percent) if the pH is to be kept within the physiologic range. Therefore, to increase the pCO_2 of the gas phase within the incubation vessel, the practice adopted was to flush the open vessels with 5 percent CO_2 : 95 percent O_2.

Although much valuable information has been gained using this technique, we were aware that the gas phase employed was actually of unknown composition and in any case represented conditions quite different from those to which the lens is exposed in vivo. Simply flushing an open vessel at atmospheric pressure with a gas mixture of known composition fails to insure that the atmosphere within the vessel actually attains the composition of the gas mixture used. In the situation outlined, one may assume only that the air within the vessel was enriched somewhat with carbon dioxide and oxygen, not that it was replaced by 5 per cent CO^2 : 95 percent O_2. Furthermore, whatever the final composition of the atmosphere, this procedure must have resulted in a much higher oxygen tension and a considerably lower carbon dioxide tension than those to which the lens is exposed in vivo, since the physiologic oxygen tension of the aqueous humor is about 50 mm Hg (approximately seven volumes percent)[4] and its CO_2 tension is roughly 40 to 45 mm (five to six volumes percent). Such a wide departure from physiologic conditions might easily result in alterations in lens metabolism. Indeed, recent cation balance studies in which the closed incubation vessel was evacuated to a residual pressure of less than 50 mm Hg before filling with the gas phase to be used showed reversal of the cold-induced cation shift to proceed more efficiently when the gas phase consisted of 5 percent CO_2 : 95 percent He, than when 5 percent CO_2 : 95 percent O_2 was used.[5]

Thus it seemed advisable to investigate further the effects of varying oxygen tensions on lens metabolism; particularly the metabolism of glucose since this sugar is the primary source of metabolic energy for the lens. If such studies were to yield valid results however, the composition of the atmosphere within the incubation vessel had to be known with a considerable degree of accuracy. Consequently, a gassing technique was developed, using an apparatus devised in our laboratory, in which nearly all air is evacuated from the sealed incubation vessel prior to passing in the gas phase to be studied. Using this technique, the role of oxygen in lenticular glucose metabolism has been studied by determining the amount of glucose taken up from the medium by intact and decapsulated lenses. The contributions of the lens capsule, with its adherent epithelium, and of the lens body to the total glucose up-

take of decapsulated lenses have also been investigated.

Experimental

Lenses

Rabbit eyes obtained from a local slaughterhouse were used throughout. Eyes were enucleated immediately after killing the animal by decapitation, immersed in saline and transported to the laboratory at ambient temperature. From this point, aseptic technique was observed and sterile equipment and solutions were used until the incubation was completed. Lenses were excised by the posterior approach as previously described[2] and placed anterior surface up in 3.0 ml. of a modified Tyrode's solution[6] carefully pipetted into a 1″ × 4″ culture tube. The tube was stoppered with a sleeve-type vaccine bottle cap and the gas phase introduced.

Gassing technique

Even if the pH of the medium is adjusted to the physiologic range prior to being transferred to the incubation vessels, carbon dioxide is lost and the pH of the medium rises upon exposure to the low pCO_2 of air. Evacuation of the vessels aggravates this situation to such an extent that a carbon dioxide concentration of five to six volumes percent in the new gas phase does not suffice to restore the desired pH (7.2 to 7.4). However, if the pH of the medium is reduced to 7.0 or a little less before evacuation, the pH does not rise so far beyond the physiologic range upon evacuation and pH 7.2 to 7.4 is readily restored by exposure to atmospheres containing five to six percent carbon dioxide. Therefore, the technique, employing the apparatus diagrammed in Figure 1 has been developed.

The gassing needle, A in Figure 1, and another needle of slightly smaller bore serving as a vent, are inserted through the cap of the incubation tube, the two-way stopcock is turned to admit gas from a tank containing pure carbon dioxide, and the tube is flushed with carbon dioxide at a pressure of approximately 100 mm Hg for 15 seconds. Both needles are then withdrawn, the evacuation needle, B, is inserted and evacuation is carried out using a water aspirator until the residual pressure within the vessel is reduced to 50 mm Hg or less. (The difference between atmospheric pressure and the height of the mercury column in the apparatus gives the residual pressure within the vessel.) When the pressure within the container has been reduced to the required level, the evacuation needle is withdrawn, the aspirator turned off and the stopcock adjusted to admit gas from a cylinder containing the mixture to be studied. The apparatus is then flushed with gas from this tank at a pressure of 100 mm Hg for at least one minute. (Over 500 ml. of gas passes through a 20-gauge needle in one-half minute at this pressure.) With the gas still flowing, the gassing needle is then inserted into the evacuated tube, a smaller bore vent needle is inserted a fraction of a second later and gas is passed through the container for three to five minutes. The needles are then withdrawn simultaneously and the vessel is transferred to the 37°C. water bath for the required period.

This gassing procedure resembles one often employed for altering gas atmospheres in Warburg flasks which is estimated to allow only about 0.1 percent of the original gas phase to remain in the flask.[7] When using air as the gas phase, the incubation system was flushed with CO_2, evacuated and gassed with a mixture containing five to six percent CO_2 to adjust the pH. The cap was removed for three to five minutes, then replaced and the incubation carried out as with the other samples.

Experimental procedure

All incubations were for a period of six hours. At the end of the incubation, the lens was removed from the vessel, blotted on a gauze pad and weighed. An aliquot of the medium was removed and a Somogyi filtrate prepared for the determination of glucose. In studies comparing intact and decapsulated

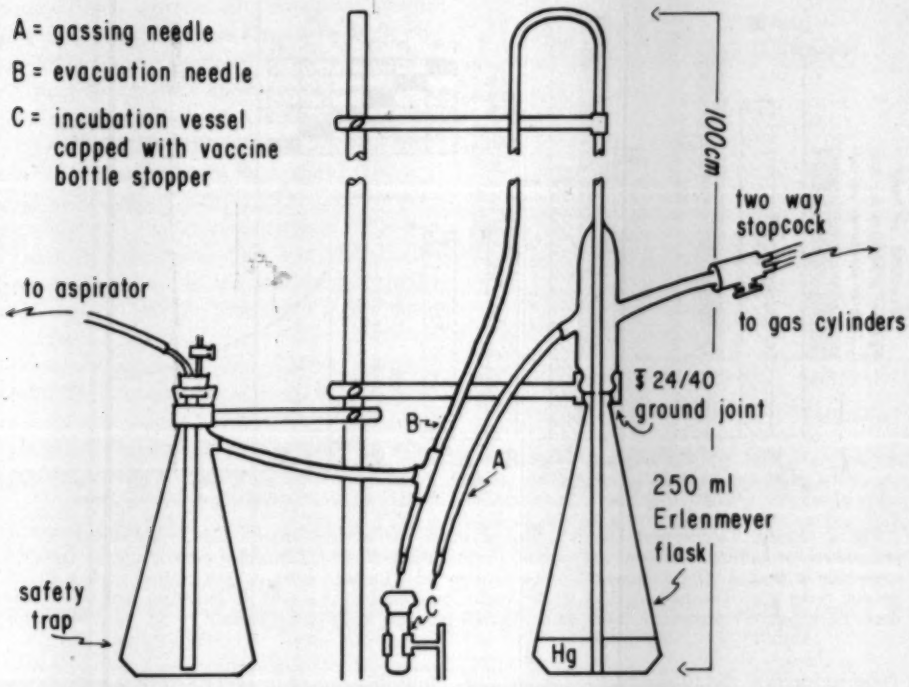

Fig. 1 (Lewis, Talman and Harris). Apparatus used for replacing the air in incubation vessels with a gas phase of known composition.

lenses, the weight of the decapsulated lens was taken to be the same as that of its intact mate. When the relative contributions of the capsule and lens body to the glucose uptake of decapsulated lenses were studied, one lens of a pair was excised, weighed on a sterile bit of aluminum foil, then decapsulated with fine forceps and both the capsule and lens body were placed in the same incubation vessel. The other member of the pair was excised, decapsulated, and the capsule placed in a vessel. The body of that lens was weighed on a sterile slip of aluminum foil, then transferred to a separate incubation vessel. The weight of the capsule of the second lens was calculated by subtracting the weight of its lens body from the weight of its intact mate.

In all studies using intact lenses, glucose was determined by the Nelson-Somogyi technique.[8] When investigating decapsulated lenses, the glucose oxidase research method described by Saifer and Gerstenfeld[9] was employed. Glucose uptake was calculated by subtracting the amount of glucose remaining in an aliquot of medium in which tissue had been incubated from that originally present in the aliquot as determined by analysis of a reagent blank treated in the same manner as the samples. For comparative purposes, the total glucose consumption was then converted to a figure expressed in terms of milligrams of glucose taken up per gram of tissue per hour.

All gas mixtures except air were prepared in steel cylinders.* Six different atmospheres were studied: 5 percent CO_2 : 95 percent He; 6 percent CO_2 : 7 per cent O_2 : 87 per cent N_2

* We wish to thank Dr. Benjamin Ross, Department of Physiology, University of Oregon Medical School, for preparing the 5 percent CO_2: 95 percent He mixture.

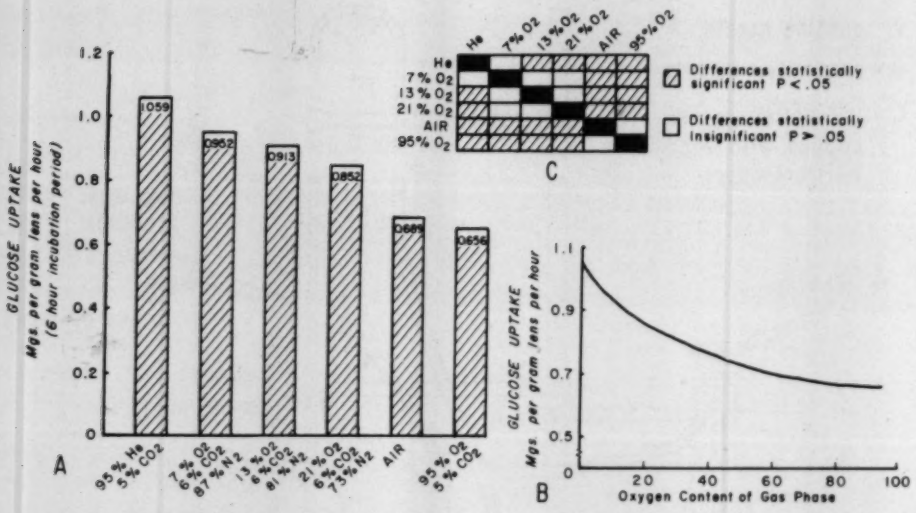

Fig. 2 (Lewis, Talman and Harris). Glucose uptake of isolated intact rabbit lenses exposed to gas atmosphere of different oxygen and carbon dioxide contents. (A) Lenticular glucose uptake under all atmospheres studied. (B) Regression of glucose uptake with increasing oxygen tension, carbon dioxide tension being approximately constant. (C) Graphic presentation of results of statistical analysis of these data. Each figure represents a mean value derived from 10 to 12 observations.

(approximating the O_2 and CO_2 tensions of the aqueous humor); 6 percent CO_2 : 13 percent O_2 : 81 percent N_2 (approximating the O_2 and CO_2 tensions of arterial blood); 6 percent CO_2 : 21 percent O_2 : 73 percent N_2 (approximating the O_2 tension of air but with CO_2 content high enough to maintain the pH of medium in the physiologic range); air itself; and the 5 percent CO_2 : 95 percent O_2 mixture used in many of the previous studies.

Results

Intact lenses

These results are summarized in Figure 2 which shows (graph A) that glucose uptake by the intact lens decreases as the oxygen content of the gas phase increases. The regression is not linear however (graph B). Actually a rather large increase in oxygen tension in required to produce a statistically significant reduction in lenticular glucose uptake. Examination of the results of statistical analysis of these data (graph C) indicate that the oxygen content of the overlying atmosphere must be increased by an increment of the order of 13 or 14 percent before a significant decrease in glucose consumption ensues. Thus, lenses incubated under anaerobic conditions (95 percent He: 5 percent CO_2) do not take up significantly more glucose than lenses exposed to an atmosphere containing 7 percent O_2 : 6 percent CO_2 : 87 percent N_2. They do, however, absorb significantly more glucose from the medium than lenses exposed to 13 percent O_2 : 6 percent CO_2 : 81 percent N_2. Similarly, the glucose uptake of lenses incubated under the 7 percent oxygen mixture does not differ significantly from that of lenses exposed to the 13 percent oxygen mixture, but is significantly greater than that of lenses overlaid with 21 percent oxygen: 6 percent CO_2 : 73 percent N_2 (incremental increase in oxygen content, 14 percent). Glucose consumption is a rather gross measure of metabolic activity, however, and it is entirely possible that significant effects of smaller increments in oxygen content could be identified by using more refined tech-

niques. Such a proposal is supported by the fact that cataracts have repeatedly been observed in animals rendered severely hypoxic[10] yet the lens normally functions at the oxygen tension exerted by 7 volumes percent oxygen. This biologically significant difference produced by a rather small increment in oxygen tension contrasts sharply with our results showing that the glucose uptakes of lenses incubated anaerobically are not statistically greater than those of lenses incubated under the 7 percent oxygen mixture. Furthermore, data from a few of our experiments in which the two members of a pair of lenses were exposed to different atmospheres indicate that such pairing might produce statistically significant differences in glucose uptake with smaller increments in the oxygen content of the atmosphere. Nevertheless, it is interesting to note that the glucose uptakes of lenses from different animals exposed to atmospheres approximating the oxygen tensions of the aqueous humor (7 volumes percent) and arterial blood (13 volumes percent) do not differ significantly. That is, lens metabolism, as measured by glucose consumption, does not appear to be affected by exposing the lens to the highest oxygen tension it is likely to encounter physiologically.

At least two other items of interest are illustrated by these data. First, air supports a significantly lower lenticular glucose uptake than an atmosphere composed of 21 percent O_2 : 6 percent CO_2 : 73 percent N_2, a mixture approximating the oxygen tension of air but having a much higher pCO_2 than air. Although this discrepancy in lenticular glucose uptake under two gas phases having nearly the same oxygen contents appears to reflect the presence of carbon dioxide, this is probably not a direct effect of carbon dioxide per se. More likely it reflects the participation of carbon dioxide in the bicarbonate: carbonic acid buffer system thus maintaining the pH at a physiologic level. Second, lenticular glucose uptake in air does not differ significantly from that occurring under 95 percent O_2 : 5 percent CO_2. This

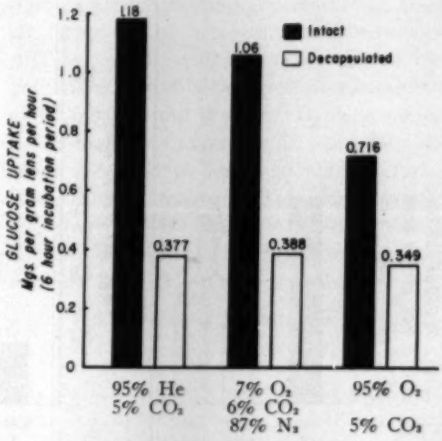

Fig. 3 (Lewis, Talman and Harris). Glucose uptake of intact and decapsulated rabbit lenses exposed to gas atmospheres having different oxygen content. 12 pairs of lenses studied under each atmosphere.

observation probably accounts for the consistent and reproducible results obtained in the previous cation balance studies.

Decapsulated lenses

In contrast to the findings with intact lenses, glucose consumption by decapsulated lenses proved to be unaffected by changes in the oxygen content of the gas phase. As illustrated in Figure 3, decapsulated lenses take up the same amount of glucose whether incubated in an oxygen-free atmosphere, in the 7 percent oxygen atmosphere, or in the 95 percent oxygen atmosphere. These results not only confirm an earlier observation made in this laboratory,[11] but actually emphasize the reduction in lenticular glucose uptake resulting from decapsulation since the earlier studies employed a high oxygen tension which has now been demonstrated to reduce glucose uptake by the intact lens.

On the basis of these data, it seems reasonable to postulate that the residual glycolytic activity displayed by decapsulated lenses is anaerobic in nature. Apparently the enzymatic mechanisms mediating glucose oxidation in the lens are so disrupted by de-

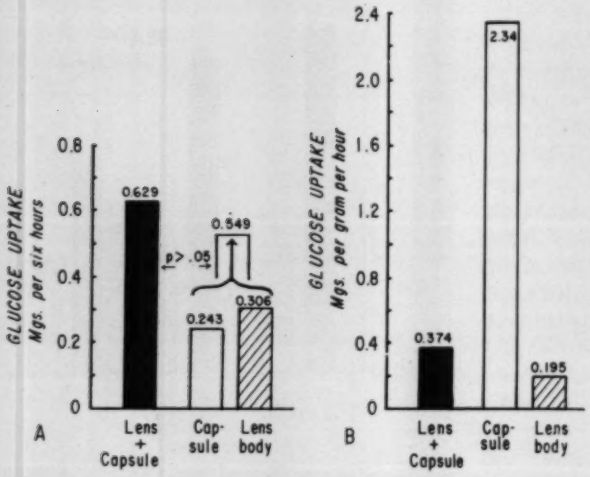

Fig. 4 (Lewis, Talman and Harris). Glucose uptake of decapsulated rabbit lenses under anaerobic conditions (5 percent CO_2 : 95 percent He). (A) Absolute glucose uptake during 6 hours incubation. (B) Rate of glucose uptake per unit weight. Both members of a lens pair were decapsulated, the capsule and body of one member being placed in the same incubation vessel while those of the other member were placed in separate vessels. 12 pairs were studied.

capsulation that they fail to function even in the presence of oxygen. Attention was then turned to determining whether this anaerobic activity was associated exclusively with the lens capsule and its adherent epithelium, with the lens body, or if the two portions exert mutually inhibitory or stimulatory effects. As may be seen from Figure 4A, both the capsular and central portions of the decapsulated lens retain some ability to take up glucose. In absolute terms, the capsular portion consumes somewhat less glucose than the remainder of the lens and neither fraction influences the glucose uptake of the other since summation of the two figures for glucose consumption yields a figure which does not differ statistically ($p>.05$) from the glucose uptake of the capsule and body of the lens mate incubated in the same vessel. To evaluate adequately the relative glycolytic activities of the two fractions, however, it is necessary to translate their absolute glucose uptakes into terms of glucose consumed per unit weight of tissue. Such figures are presented in Figure 4B which shows that the capsule, with its adherent epithelium, is about 12 times as active as the lens body. Thus, the residual glycolytic activity of the decapsulated lens is not associated exclusively with either the capsular or central portion since each part consumes roughly one-half the total uptake of glucose. However, this consumption is accomplished by a much smaller mass of tissue in the case of the capsular portion, indicating that this portion contains the enzymes mediating anaerobic glycolysis in rather high concentration.

Discussion

Decreased glucose uptake by intact lenses as a result of exposure to high oxygen tensions may be interpreted as an example of either or both of two well-established biologic phenomena, oxygen toxicity and/or the Pasteur effect. The former interpretation gains credence from the fact that the lens functions at such a low oxygen tension in vivo. However, extensive studies of retrolental fibroplasia, an ocular disorder known to result from exposure to unphysiologically high oxygen tensions, provide evidence to the contrary.[12] If high concentrations of oxygen actually poison the lens in some way, it seems that cataracts might conceivably constitute a part of the syndrome of retrolental fibroplasia and this has not been found to be the case. Thus, it seems unlikely that reduced lenticular glucose uptake at high oxygen tensions arises from a poisoning action of oxygen.

With regard to the Pasteur effect, this phenomenon is defined as an inhibition of glycolysis by oxygen.[13] Hence, the inverse relationship between glucose uptake and oxygen tension observed in intact lenses falls, by definition, into this category. Recognition of this fact is of little value in definitely identifying the metabolic changes occurring in lenses exposed to high oxygen tensions however, since the mechanism(s) operating to produce the Pasteur effect have not yet been completely elucidated. Nevertheless, one of the hypotheses advanced to account for this phenomenon, the proposal that increasing oxygen tensions promote the direct oxidation of glucose via the hexose monophosphate shunt (also known as the phosphogluconate oxidation pathway) is of special interest when considering lens metabolism. Kinoshita[14] and Kinoshita and Wachtl[15] have shown that the lens differs from other tissues in producing carbon dioxide from glucose primarily via the hexose monophosphate shunt with little, if any, participation by the tricarboxylic acid cycle. Thus the observed reductions in glucose uptake by intact lenses exposed to high concentrations of oxygen lends support to the hypothesis that increased activity of the phosphogluconate oxidative pathway underlies the Pasteur effect.

This explanation of the Pasteur effect assumes that the metabolic change resulting from high oxygen tensions consists of the acceleration of a normal pathway for the aerobic utilization of glucose. In the case of the lens, which Kinoshita and Wachtl[15] have found to utilize about 10 percent of its glucose via the phosphogluconate oxidative pathway and to catabolize the remaining 90 percent anaerobically to lactic acid, an event of this type represents a shift toward a more aerobic type of metabolism from a predominently anaerobic one. Thus possible deleterious effects of elevated oxygen tensions on the lens may stem from a shift in the relative proportions of the total lenticular energy requirement fulfilled by the two normally functioning energy-yielding processes, not from the induction of some abnormal metabolic process. Again, evidence favoring this hypothesis is found in the work of Kinoshita[14] and Kinoshita and Wachtl.[15] By determining the radioactivity appearing in the carbon dioxide produced by lenses incubated in glucose-1-C^{14} as a measure of phosphogluconate oxidative activity and glucose-6-C^{14} as a measure of tricarboxylic acid activity, these workers have shown that the ratio:

$$\frac{C^{14}O_2 \text{ from glucose-1-}C^{14}}{C^{14}O_2 \text{ from glucose-6-}C^{14}}$$

was the same in different experimental situations employing 95 percent and 7 percent oxygen atmospheres. Hence the mode of lenticular glucose oxidation appears not to be influenced by oxygen tension.

These studies, then, like the observations made in studies of retrolental fibroplasia, provide no evidence that high oxygen tensions actually poison the lens, although that possibility cannot yet be unequivocally excluded. It seems more logical, however, to postulate that reductions in lenticular glucose uptake resulting from exposure to elevated oxygen tensions simply reflect a stimulation of aerobic glucose metabolism, a process yielding roughly 12 times as much energy per mole of glucose as anaerobic glycolysis. Nevertheless, in attempting to elucidate the metabolic patterns normally operating to preserve the structural and functional integrity of the lens, it is important that the relative amounts of glucose metabolized via the aerobic and anaerobic routes approximate those occurring in vivo. Therefore, a physiologic oxygen tension should be employed for in vitro studies of lens metabolism whenever possible.

The observation that the hexose monophosphate shunt and the Embden-Meyerhof scheme of anaerobic glycolysis are probably the two pathways by which the lens normally utilizes glucose is also significant in accounting for our findings with decapsulated lenses. To be sure, the greatly reduced glucose up-

take of apparently anaerobic nature displayed by decapsulated lenses could arise from a necessity for preserving the structural relationships existing in the intact lens if all enzymes are to function properly. However, the enzymes of both the hexose monophosphate shunt and the Embden-Meyerhof pathway are soluble and may be assumed to leak out into the surrounding medium, along with various cofactors, when the lens capsule is removed. The resultant dilution of both the enzymes and cofactors may easily account for the reduced glucose uptakes of decapsulated lenses. As indicated previously, phosphogluconate oxidative activity in the lens is not very great under physiologic conditions. This observation suggests that the enzymes mediating this process are present in the lens in rather low concentration and makes it easy to conceive of dilution with a medium as simple in composition as that used in these studies resulting in complete cessation of phosphogluconate activity. It is entirely possible that fortification of the medium with appropriate cofactors, for example, triphosphopyridine nucleotide (TPN) would restore phosphogluconate oxidative activity.

Anaerobic glycolysis on the other hand, proceeds very actively in the lens, indicating that the enzymes and cofactors involved in this process are probably present in relatively high concentration. Such an assumption is supported by the observation that decapsulation sharply reduces, but does not abolish, anaerobic glycolytic activity in the lens. Here again, fortification of the medium with appropriate cofactors might lead to partial or complete restoration of enzymatic activity.

The high anaerobic glycolytic activity per unit weight associated with the lens capsule and probably due to the adherent epithelium is extremely interesting. On the basis of its topographic location on the anterior surface of the lens adjacent to the nutrient aqueous humor, one might logically presume that this portion of the lens would display a relatively aerobic type of metabolism. Instead, these experiments offer evidence for great anaerobic glycolytic activity in this portion of the lens. This observation, in turn, suggests that the energy expended in transporting materials across the lens capsule is derived chiefly from anaerobic glycolysis. Such an hypothesis is supported by the observation that iodoacetate prevents recovery from the cold-induced cation shift[16] and that such recovery proceeds more efficiently under anaerobic conditions.[5]

Demonstration of an inhibition of lenticular glucose uptake as a result of exposure to atmospheres of high oxygen content raises a question regarding the validity of previous findings obtained using gas phases rich in oxygen. These findings are probably not negated by the results of the present studies, however, since the best evidence indicates that high oxygen tensions do not poison the lens but rather only stimulate a normal aerobic process. Nevertheless, it seems advisable to choose and control with care the gas phase used for in vitro studies of lens metabolism.

Summary

1. Increases in the oxygen content of the overlying atmosphere, with the pCO_2 (and hence the pH of the medium) held nearly constant, result in decreases in the glucose consumptions of isolated intact rabbit lenses.

2. Decapsulated lenses take up much less glucose than their intact mates and this residual glucose uptake appears to be accomplished by an anaerobic mechanism since decapsulated lenses consume the same amount of glucose whether incubated anaerobically or aerobically.

3. Both the capsule, with its adherent epithelium, and the body of the lens participate in this anaerobic glucose consumption of decapsulated lenses but the former is far more active, per unit weight, than the latter.

4. The inverse relationship between glucose consumption and oxygen tension observed with intact lenses constitutes an example of the Pasteur effect and probably does not arise from a toxic action of oxygen.

It is pointed out that the demonstration of a Pasteur effect in the lens, which oxidizes glucose primarily via the hexose monophosphate shunt, lends support to the hypothesis that the Pasteur effect reflects a stimulation of the direct oxidation of glucose via that route.

6. It is also suggested that the abolition of glucose oxidation and the marked reduction in anaerobic glycolysis occurring as a consequence of decapsulating the lens result, at least in part, from dilution of the enzymes and cofactors mediating these reaction sequences.

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Discussion

Dr. Carl Wachtl (Detroit): The results of this investigation demonstrate the importance of an exact control of the gas phase used in lens culture. They also show that the lens consumes more glucose at a lower oxygen tension, that is when conditions are more similar to those in vivo.

A decreased glucose consumption is not necessarily harmful, as Mr. Lewis pointed out, if the energy requirements of the lens are met, for instance by metabolizing the nutrient via a pathway where the same amount of energy is obtained from a smaller amount of glucose.

Mamo and Leinfelder determined the effect of oxygen tension on growth characteristics of human lens epithelium in culture and also found that oxygen above 20 percent was toxic and that the toxicity was proportional to the oxygen concentration. Anaerobiosis and an oxygen concentration of much less than 20 percent, however, were not conducive to growth.

In this study, rabbit lenses were cultured for 6 hours. I would like to ask Mr. Lewis whether he also tried to use longer periods of culture—say, 24 hours. There is a possibility that effects of

smaller increments in oxygen concentration would then become more pronounced.

Glucose consumption of lenses over short periods may also not be entirely uniform, since it takes the lens some time to become accustomed to its surroundings. We found that to be true in the case of the mitotic activity of cultured lenses where the number of epithelial cells was depressed after two hours but returned to normal after six hours. This was in confirmation of a similar observation made earlier by Dr. Constant.

It would be useful to take into consideration the difference in the sugar content of lenses before and after culture, and to distinguish here between glucose and fructose. Incidentally, as far as decapsulated lenses are concerned, we also observed that there was a decrease in glucose consumption in the few instances when we cultured such lenses.

In order to gain more information concerning the possible mechanism of the Pasteur-like effect observed at high oxygen concentrations, it might be advisable to measure the formation of metabolic end products such as lactic acid and CO_2 at different oxygen concentrations. It is my hope that the authors will pursue this problem further, and I wish to commend them on their good work.

DR. ZACHARIAS DISCHE (New York): I would like to ask the authors whether they really considered the possibility that a great part of the oxygen consumption of the whole lens may be due not to real respiratory processes but to some purely nonreversible oxidation of some reducing substances in the lens fibers.

I think the fact that in the presence of oxygen the utilization of glucose dropped only by about 20 to 25 percent, and that this phenomenon was not observed after decapsulation, suggests that in measuring the oxygen uptake of the whole lens we are dealing with two different processes, one of which is a real respiratory process, which however is almost confined completely to the epithelium and which shows a significant Pasteur effect. The other may be due to oxidation, for instance, of glutathione in the lens fibers, which may not display any real oxidative respiratory processes.

This is an interesting problem from still another point of view, namely, that if really the shunt mechanism is a predominant form of oxidation in the lens and even in the lens epithelium, then the problem arises (and it was widely discussed) as to whether the shunt mechanism produces a Pasteur effect. This is not quite clear, because undoubtedly the Pasteur effect will have something to do with the suppression or limitation of the phosphorylation of glucose.

The dominant concept now is that such oxidative processes which use TPN as intermediate nucleotide are not able to contribute to aerobic phosphorylation, and under these conditions it might be doubtful whether they can contribute or produce any Pasteur effect.

From this point of view, therefore, I think the measuring of the effect in the capsule itself—that is, the measuring of the effectiveness of the suppression of glucose consumption by the capsule itself—would be of great theoretical interest.

DR. ELLEN L. TALMAN (closing): With the permission of the chairman I would like to close for Mr. Lewis.

I would like to answer Dr. Wachtl's very kind discussion with reference to prolonging the incubation period. We did this. We incubated lenses for 24 hours under the 95-percent helium-five percent CO_2 mixture and under the 7 percent oxygen mixture (the physiologic one). This experiment did not produce any qualitative difference at all in our results. The lenses incubated under 95 percent helium still took up more glucose than the ones incubated under seven percent oxygen.

The only difference was that the rates of uptake were reduced considerably, probably because our medium did not have quite enough of the co-factors needed to maintain mitosis for that long a period. That would be my guess, and it is simply a guess.

As far as discussing what goes on so far as oxygen uptake is concerned we don't care to enter into that discussion. We were not measuring oxygen uptake. We were simply studying the effect of oxygen in the atmosphere on glucose uptake, so I think our data are not sufficient to permit us to enter into any consideration of this point at present.

THE EVOLUTIONARY SIGNIFICANCE OF LENS ORGAN SPECIFICITY*

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New York

Cross-reaction of vertebrate lens antibodies with lenses from other widely divergent vertebrates is well established. This peculiar immunologic behavior of lens tissue is called organ specificity. With most other tissues, species specificity predominates and cross reactions occur only in very closely related species.[1]

The organ specificity of lens antibodies was first recognized by Uhlenhuth[2] in 1903 and later confirmed by many other investigators.[3,4,5] At that time and until recently, the methods available could only establish the absence or degree of cross-reaction.[6] Because these methods do not allow the determination of the number of immunologic components involved in such reactions, attempts to correlate chemical or biologic factors with the degree of cross reactivity become equivocal.[7,8]

Introduction of the two directional agar diffusion techniques[9] has shown the presence of multiple antigen-antibody systems in the reaction of lens and its antisera.[10] Our laboratory has demonstrated the presence of a minimum of six distinct components in this system.[11,12,13]

More recently Francois[14] and Witmer[15] have shown that Grabar's immunoelectrophoretic method[16] is even more informative. Precise knowledge of the number of immunologic components involved is of paramount importance in establishing a biologic or chemical basis for organ specificity and is the aim of the experiments presented in this paper.

Materials and Methods

The animal lenses used were obtained as soon as possible after killing the animal and quickly frozen. Human cataract lenses were collected immediately after surgery, and placed into dry ice containers. The lenses were weighed wet and ground with a teflon glass homogenizer (A. H. Thomas, Phila.) to a concentration of 100 mg./ml. wet weight in sterile 0.85 percent NaCl. A large enough pool was prepared for the entire study, stored in appropriate aliquotes in the deep freeze at −25°C and withdrawn as needed.

For immunoelectrophoresis, the lens homogenates were centrifuged for one hour at 20,000 rpm in the cold in the Spinco ultracentrifuge (rotor no. 21). The clear solution containing the soluble lens components was then lyophilized.

Such lens extracts were obtained from the following specimens: Pooled human cataract, Rhesus (Macaca rhesus), and Java (Macaca java) monkeys, bovine (Bos taurus), rabbit (Oryctolagus cuniculus), guinea pig (Cavia porcellus), rat (Rattus norveqicus), mouse (Mus musculus), whale (Balaenopteria borealis), chicken (Gallus domesticus), frog (Rana pipiens), menhaden (Brevoortia tyranus), carp (Cyprinus carpia), squid (Loligo paelei), and lobster eye-stalks (homarus vulgaris).

Immunization

The prepared lens material was homogenized with Freund's adjuvant[17] in the following way:

Lens homogenate 100 mg. wet weight/ml.	10 ml.
Bayol F (Esso)	8.5 ml.
Arlacel A (Atlas)	1.5 ml.
Mycobacterium butyricum (heat killed and lyophilized)	20 mg.

Adult chinchilla rabbits (five to six pounds) were used for immunization. 1 ml. of the lens homogenate adjuvant was injected intradermally into six widely separated sites on each rabbit. The dose per rabbit for each immunization was 50 mg. of wet weight of

* This investigation was supported by a research grant from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

lens homogenate. This represents roughly 15 mg. of protein/dose. The insoluble material, quantitatively differing in each lens species was included. The first few injections were given every 14 days, and the later ones every month. The antisera were collected from the central ear artery 10 days after immunization and stored aseptically in the cold. Most sera proved of rather high potency after four to six doses. The rabbits were maintained under immunization for periods up to two years.

All immune sera were tested for the presence of antibodies against other tissue components. Such nonlens components were found only in the antifrog lens serum. These were removed by absorbing the sera with various lyophilized tissues and after 24 hours at 4.0°C, centrifuging off the precipitate at high speed. This process was repeated until no further precipitation occurred.

Concentration of the antibodies was obtained by precipitation of the globulin fraction of the immune sera with saturated $(NH_4)_2SO_4$ (1:1), followed by centrifugation at 40,000 rpm in the Spinco ultracentrifuge (rotor no. 40) for one hour. The sediment obtained was dissolved in 0.05 M phosphate buffer at pH 7.85, containing 0.3 M glycine. All these concentration procedures were carried out at 4.0°C.

Immunoelectrophoresis

This method was based on the micro technique described by Scheiddiger.[18] Thoroughly cleaned microscopic slides (1″ × 3″) were covered each with 2.5 ml. of 2.0 percent Bacto agar (Difco) solution in veronal acetate buffer pH 8.2 and ionic strength 0.05.

The dimensions of the antigen well and serum trench, as well as the distance between them, were cut and located so that optimum conditions for visualizing all components were first established. The dimensions were chosen as optimal for these systems using a soluble antigen concentration of 20 mg. protein/ml. The antigen well was 2.0 mm. in diameter, while the serum trench was 3.0 mm. wide and 37 mm. long. The distance from the edge of the well to the edge of the trench was 2.5 mm. The protein concentration was determined by the method of Kalkar, et al.[19] The immunoelectrophoresis procedure was carried out at 4.0°C, and 6.5 volts/cm were applied across the agar for 150′. The slides were then placed in a moist chamber to prevent evaporation and kept at 4.0°C. Photographs of the developing precipitin lines were taken after one day and at intervals up to 14 days.

Results

Figure 1 illustrates the results obtained with the antihuman cataract lens serum against different vertebrate lenses. The immunoelectrophoretic patterns obtained are represented by the tracings shown. The number of precipitin lines thus obtained has been arbitrarily divided into three groups, depending on the position of the centers of precipitin arcs developed. These three groups represent approximately the minimum number of components which correlate with the classical lens protein fractions, namely the "alpha," "beta" and "gamma" crystallines. In a few cases the division between beta and

REACTION OF ANTIHUMAN (CATARACT) LENS SERUM WITH DIFFERENT VERTEBRATE LENSES

LENS	No. of PRECIPITIN LINES IN DIFFERENT MOBILITY GROUPS α	β	γ	TOTAL NO. OF PRECIPITIN LINES	IMMUNOELECTROPHORETIC PATTERNS
HUMAN	2	4	2	8	
RHESUS	2	5	3	10	
JAVA	2	5	3	10	
BOVINE	2	5	2	9	
RABBIT	2	4	3	9	
G. PIG	2	4	3	9	
RAT	2	4	3	9	
MOUSE	2	4	3	9	
WHALE	2	5	2	9	
CHICKEN	2	3	2	7	
FROG	2	3	1	6	
MENHADEN	1	3	1	5	
CARP	1	3	1	5	
SQUID	0	0	0	0	
LOBSTER	0	0	0	0	

Fig. 1 (Manski, et al.). Reaction of antihuman (cataract) lens serum with different vertebrate lenses.

gamma lines was rather difficult to make.

The electrophoretic patterns of the antihuman cataract lens serum represents development of precipitin lines after two days. Development of these lines for a longer period of time caused striation effects which are difficult to interpret. The precipitin lines of the antirabbit lens serum developed much more slowly and after 14 days are comparable with the antihuman cataract lens electrophoretic patterns as judged by the arcing of the precipitin lines.

The antirabbit lens sera showed fewer precipitin lines with all the lenses tested, than did the antihuman cataract lens serum. In spite of this difference, similar overall trends were seen with respect to the decreasing number of components which occurred as one descends from higher to lower vertebrate species (fig. 2).

To show that this is not merely the result of a simpler composition of the chicken, frog and fish lenses, these were tested against their respective antisera, and the results are presented in Figure 3. It may be seen that the number of components are approximately the same as those found in antimammalian lens-mammalian lens reactions.

REACTION OF ANTIRABBIT LENS SERUM WITH DIFFERENT VERTEBRATE LENSES

LENS	NO. of PRECIPITIN LINES IN DIFFERENT MOBILITY GROUPS α	β	γ	TOTAL NO. OF PRECIPITIN LINES	IMMUNOELECTROPHORETIC PATTERNS
HUMAN	2	2	0	4	
RHESUS	2	2	1	5	
JAVA	2	2	1	5	
BOVINE	2	2	0	4	
RABBIT	2	2	0	4	
G.PIG	2	2	0	4	
RAT	2	2	0	4	
MOUSE	2	2	0	4	
WHALE	2	2	0	4	
CHICKEN	1	1	0	2	
FROG	1	1	0	2	
MENHADEN	1	0	0	1	
CARP	1	0	0	1	
SQUID	0	0	0	0	
LOBSTER	0	0	0	0	

Fig. 2 (Manski, et al.). Reaction of antirabbit lens serum with different vertebrate lenses.

HOMOLOGOUS REACTION OF SOME ANTIVERTEBRATE LENS SERA

LENS	Rabbit immune serum against lens of	IMMUNOELECTROPHORETIC PATTERNS
FISH	FISH	
FROG	FROG	
CHICKEN	CHICKEN	
RABBIT	RABBIT	
HUMAN	HUMAN	

Fig. 3 (Manski, et al.). Homologous reaction of some antivertebrate lens sera.

After absorption of antihuman cataract lens serum with mammalian, bird, amphibian and fish lenses, it was found that the cross-reactions in each instance were removed from species lower in the phylogenetic scale. Some of these results are shown in Figure 4.

Discussion

The results obtained with antihuman cataract lens serum indicate that organ specificity is more complex and involves many more components than has been assumed until the present. Contrary to earlier data and in agreement with Witmer,[15] our results show that cross-reactions occur within all three of the lens crystallines, alpha, beta and gamma.

All mammalian lenses reacting with antihuman cataract lens serum showed a similar pattern indicating the presence of nine distinct components. In the alpha crystalline region two components were found while four were found in the beta crystalline region and about three in the gamma crystallines. Only the homologous reaction of the antihuman cataract lens system showed a different pattern. It seems not unlikely that in the human cataract lens, there are some amounts of altered proteins. Only eight components could be distinguished with certainty since the patterns were quite fuzzy, possibly due to the overlapping of native protein—antinative protein and pathologic protein—antipathologic protein systems.

The nine distinct components obtained

REACTION OF DIFFERENT VERTEBRATE LENSES WITH ABSORBED ANTIHUMAN (CATARACT) LENS SERUM

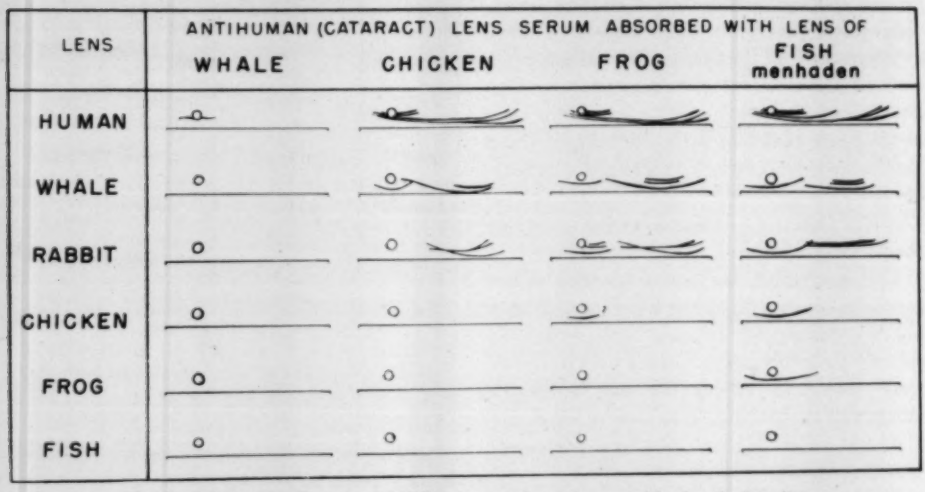

Fig. 4 (Manski, et al.). Reaction of different vertebrate lenses with absorbed antihuman (cataract) lens serum.

with all other mammalian lenses can be accepted as the minimum of components involved in organ specificity. That this number is a minimum is illustrated by the Java and Rhesus monkey lenses which clearly showed 10 cross-reacting components with antihuman cataract lens serum.

The nine to 10 mammalian factors cross-reacting with antihuman cataract lens serum decreases to seven with chicken lens, to six with frog lens and to five with fish lens. The decreasing number of components involves only the "beta" and "gamma" crystalline groups; however, in the case of the fishes, that is the lowest region of the phylogenetic sequence of the vertebrates, it involves the "alpha" crystallines as well. No cross-reaction whatsoever were found with the squid or lobster lenses. When the rabbit antirabbit lens sera were employed, the number of lens components detectable decreased from four mammalian, to two chicken and frog, and to two fish components. There is again no reaction with the squid or lobster lenses.

It is evident that the decreasing number of components found above is not caused by a simpler lens composition of the more primitive species. It was shown above that the lens of these species reacting with their own antisera showed practically the same number of components as did the mammalian lenses.

Figure 5 shows the relative position on the evolutionary scale of the species used in our investigation thus far.[20] If organ specificity has significance one should expect stepwise addition of cross-reacting components from lower to higher forms. On this basis, as examples, it should be possible to detect fish components in the amphibian lens and fish and amphibian components in the bird lens, but no bird components in the fish or amphibian lens. The results presented in Figure 4 are in agreement with this hypothesis. Absorption of antihuman cataract lens serum with whale lens removed antibodies against bird, frog and fish lenses. Absorption of the sera with chicken lens removed antibodies against frog and fish lens; and absorption with frog lens removed antibodies against fish lens. In each instance reactions were still obtained with lenses from species higher in evolution than that used for absorption.

The origin of organ specificity would then seem to lie in the retention by the lens of its

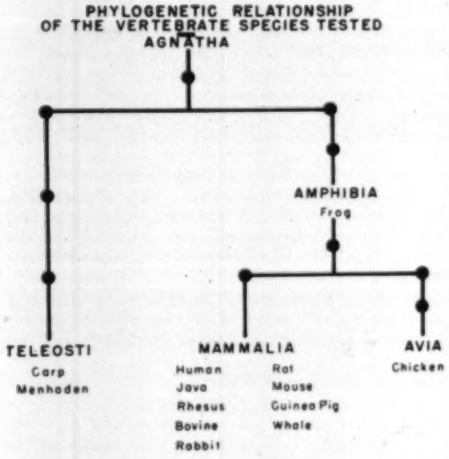

Fig. 5 (Manski, et al.). Phylogenetic relationship of the vertebrate lenses tested.

evolutionary history. The very early isolation of the lens in fetal development and its relative separation from the rest of the body may partly explain the slower evolution of this tissue in comparison to other tissues in the same species. These studies are being continued in our laboratory with lenses from many more species, to further elaborate these data.

Summary

1. Cross reactions between lenses of different vertebrates and rabbit antihuman lens (cataract) and rabbit antirabbit lens sera have been studied by immunoelectrophoresis. It was shown that the organ specificity of the lens is complex and involves nine to 10 components in the mammalian lenses.

2. Immunoelectrophoresis of different vertebrate lenses with antihuman cataract lens as well as antirabbit lens sera showed that the number of components common between different vertebrate lenses, decrease according to the phylogenetic sequence of the vertebrate species.

3. Absorption of antihuman lens serum with appropriate vertebrate lenses removed antibodies against all lens components of species which were lower in the evolutionary scale. Cross-reactions were still obtained with lenses which were higher in evolution than that used for absorption.

4. The origin of organ specificity of the lens may lie in the retention by the lens of molecular structures acquired during successive stages of evolution.

College of Physicians and Surgeons, Columbia University (32).

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Discussion

Dr. Carl Wachtl (Detroit): The authors have demonstrated how complex the organ specificity of the lens is, and have shown that the number of proteins which lenses of different species have in common increases with advancing evolutionary level. When antihuman (cataract) lens serum was used with different vertebrate lenses, the sum of the precipitin lines within the class of mammals was 9 to 10, while it was 4 to 5 in the case of antirabbit serum. I wonder why cataractous instead of normal antiserum was employed. Protein changes in mature cataract could be the reason for the difference in the sum of components observed.

Homologous reactions of some antivertebrate lens sera demonstrated that, for instance, fish lenses had a similar number of protein components as mammalian lenses. Rabbit lens, however, when reacting with rabbit immune serum, showed only four lines. Do the authors think there might be lens proteins with borderline configurations in the rabbit that do not give rise to antibodies in rabbit serum, or was this perhaps due to a lower sensitivity of a particular animal or animals?

The apparent retention by the lens of its evolutionary history as observed in the species investigated may be due to its early isolation in fetal development, as suggested by the authors, assuming the embryo passes through successive evolutionary stages in its development. This separation, though, is by no means complete. The lens capsule is quite permeable to many intermediates, and it is a metabolically active organ which remains in some contact with the rest of the organism.

Isolation of the different antigenic components would permit a determination of their constituent amino acids and perhaps amino acid sequence, and thus information could be obtained as to the actual differences between these protein components. The antisera could also be an acid in testing the purity of protein fractions from the lens.

An example, that a knowledge of the antigenicity of lens proteins is also of clinical importance was given some years ago, in 1952, when injections of fish lens proteins were hailed as a so-called cure for cataract. The endophthalmitis phacoanaphylactica which resulted in some cases could have been avoided.

The authors made a significant contribution to our knowledge concerning the immunologic specificity of lens proteins. I appreciate the opportunity of discussing their paper.

Dr. W. Manski (closing): The difference in behavior between rabbits immunized with rabbit lenses and rabbits immunized with human lenses is the difference in behavior between hetero-immunization and auto-immunization. This is a large field, but evidently from our experience it seems that with auto-immune processes the antigenicity of the same components is different in the hetero-immunization.

As we have been invited to make some comments on some possibly practical or clinical consequences of the experiments we have concluded, I think there may turn out to be some clinical consequences also from this line of research.

From our immune electrophoretic evidence we see that the amount of antigens present in lenses is a minimum of 10 different. If it comes out in the pathologic situation or under surgery to a situation where auto-immunization in patients can take place, the organism has a choice of ten different antigens to produce antibodies.

It is a well-known fact in immunology that animals respond stronger to antigens which are further in their evolutionary scale. So, I could not exclude the possibility that in auto-immune processes in humans the antigenicity of components of proteins which are farther along in the evolutionary scale is greater than that of components which are closer to the group of mammalians, and that these components which are evolutionary in order ought to play a greater role in clinical auto-immune processes than the other components.

We used human cataract lenses because they are much easier to obtain than are normal lenses, and still with cataract lenses we were able to demonstrate the evolutionary dependence of the organ specificity in which we were interested.

We have used in our investigation anti-mammalian lens sera. This means anti-lens sera against the highest forms in evolution. Using this reagent, we were able to test only the presence and the stepwise addition of the most stable evolutionary proteins—that means the proteins which reach the state of the mammals.

It is still possible that not only a stepwise addition has taken place in evolution, but that some components which were transferred to the next species were later lost, and by using antilens sera against all the species which typify steps in evolution, we think we will be able to confirm that the reaction of, let's say, anti-lamprey serum with shark lenses is stronger than with human lenses, and that not only a stepwise addition has been obtained but that also some components have been lost in evolution. That brings us to the problem of the evolutionary stability of the proteins.

From all the experience of comparative serology or immunology we know that proteins from different organs show different ranges of cross-reactivity, and it is known that if we can demonstrate for the lens a cross-reaction between the most primitive vertebrates and the highest vertebrates, then by using serum it is possible to demonstrate only cross-reaction in the group of mammals but not between serum of mammals and other species.

Again, if we would use rat cells or protein components of rat cells we would be able to demonstrate the existence of cross-reaction only in the range of humans and higher apes.

So, on a molecular level there seems to exist differences in evolution of proteins obtained from different organs of the same organism, and in the instance of the lens we are in the fortunate situation that we can follow the evolution to the beginning of the vertebrate species.

THE EFFECT OF PLASMA ON GLUCOSE UPTAKE OF ISOLATED RAT LENSES*

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Cleveland, Ohio

Glucose uptake by isolated rat lenses incubated in Tyrode's solution containing 100 mg. percent, is increased when insulin is injected into the animals prior to killing them and to removal of the lenses.[1,2,3] On the other hand the addition of insulin directly to the incubation medium has no effect on the glucose uptake.[1,2] This suggests that the action of insulin requires either a modification of insulin, the presence of one or more other substances to act with insulin, or the release of a new active substance by insulin. Each of these hypotheses implies a need for some organ other than the lens for insulin action. Studies of the insulin effect following the removal of various organs and combinations of organs indicate that the liver is this essential organ;[4] although glucose uptake is increased following adrenalectomy, current unpublished studies indicate that the action of insulin is not mediated through this organ. These studies further suggest that an active substance might be isolated from the blood as it is transported to the lens. It is the purpose of this paper to provide data demonstrating that following the injection of insulin and not before, there is present in the blood a substance which will stimulate the uptake of glucose by the isolated rat lens. Some of the characteristics of this substance are described.

* This investigation was supported by Grant Number A-154 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

Experimental

In all experiments lenses of normal male Sprague-Dawley rats, obtained and handled in the manner previously described[3] were incubated for two hours in Tyrode's solution containing 100 mg. percent glucose and varying amounts of blood plasma. The glucose uptake of these lenses was calculated after determining (by the method of Somogyi[5]) the glucose remaining in the medium at the end of two hours.[2]

Plasma was obtained from male Sprague-Dawley rats weighing 120 to 150 gm. The rats were anesthetized by intravenous injection of 6.6 mg. Nembutal Na/kg. of body weight and approximately 2.0 ml. of blood were withdrawn from the surgically exposed inferior vena cava with a heparinized syringe. The blood was transferred into heparinized test tubes and kept at O°C. The cells were removed by centrifugation at 2,500 rpm for 15 minutes. In those experiments in

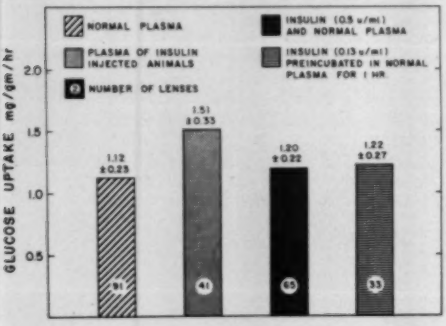

Fig. 1 (Farkas, et al.). Effect of plasma and insulin on glucose uptake of isolated rat lenses. 1.0 ml. plasma was added to 99 ml. Tyrode's solution and 0.2 ml. of the mixture was used as the incubation medium. Insulin was added as indicated.

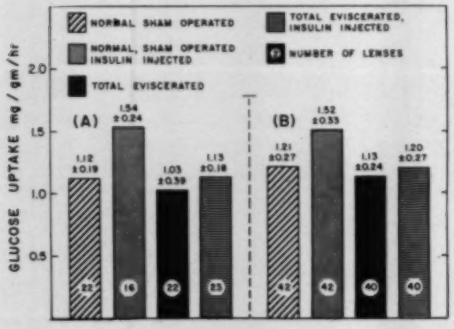

Fig. 2 (Farkas, et al.). Effect of total evisceration on glucose uptake of isolated rat lenses. (a) Lenses of insulin injected and operated animals were incubated in Tyrode's solution (data from reference 4). (b) Lenses of normal animals incubated in plasma obtained from insulin injected and operated animals. (Plasma was diluted 1:100 with Tyrode's solution.)

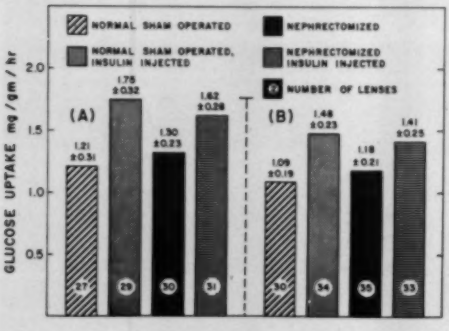

Fig. 3 (Farkas, et al.). Effect of nephrectomy on glucose uptake of isolated rat lenses. (a) Lenses of operated and insulin injected animals were incubated in Tyrode's solution (data from reference 4). (b) Lenses of normal animals incubated in plasma obtained from insulin injected and operated animals. (Plasma diluted 1:100 with Tyrode's solution.)

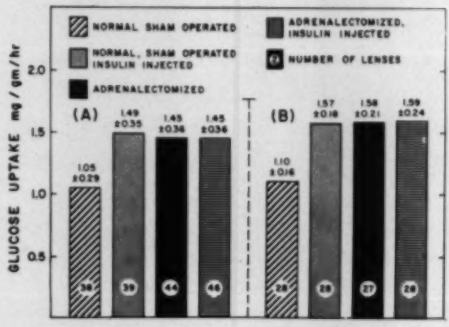

Fig. 4 (Farkas, et al.). Effect of adrenalectomy on glucose uptake of isolated rat lenses. (a) Lenses of operated and insulin injected animals were incubated in Tyrode's solution (data from reference 4). (b) Lenses of normal animals incubated in plasma obtained from insulin injected and operated animals. (Plasma diluted 1:100 with Tyrode's solution.)

which larger amounts of plasma were used the blood samples were pooled before centrifugation. When plasma from animals injected with insulin was used, 20 units of the hormone were injected into a tail vein one hour before drawing the blood sample.

Removal of the abdominal organs was carried out as previously described.[4] After surgery 20 units of insulin were injected into the surgically exposed inferior vena cava of some of the operated animals. One hour later about 1.5 ml. of blood was withdrawn from the inferior vena cava and the plasma was obtained in the manner described. Plasma of sham operated animals was obtained to serve as controls.

Results

The data are presented in Figures 1-9. The data on the glucose uptake of lenses obtained from operated animals (Series A in

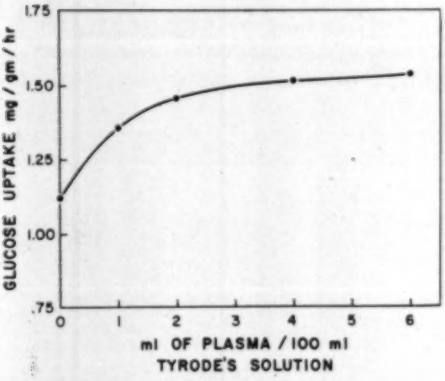

Fig. 5 (Farkas, et al.). Effect of increasing concentrations of plasma on glucose uptake of isolated rat lenses. Plasma was obtained from blood drawn 15 minutes after intravenous injection of 20u of insulin. Each point on the curve represents the average of approximately 30 lenses.

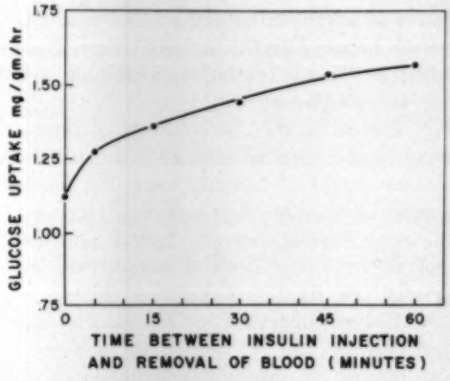

Fig. 6 (Farkas, et al.). Effect of increasing the time interval between insulin injection and removal of the blood on the ability of plasma to stimulate glucose uptake of isolated rat lenses. Plasma was obtained from blood drawn at various time intervals following intravenous injection of 20u of insulin and was diluted 1:100 with Tyrode's solution. Each point on the curve represents the average uptake of 25-30 lenses.

Figures 2-4) are those previously reported[4] and are presented here for purposes of comparison.

The following results should be noted:

1. The glucose uptake of normal lenses was significantly increased ($P < 0.001$) by the addition of 1 ml. of plasma, obtained from insulin injected animals, to 99 ml. of medium. This increase was not observed when insulin either alone or together with plasma from noninjected animals was added to the medium (fig. 1).

2. Plasma from animals subjected to total evisceration (removal of stomach, intestines, pancreas, spleen, kidneys, adrenals and liver) prior to the injection of insulin did not stimulate glucose uptake of normal lenses. This is consistent with the previous finding that insulin injection into eviscerated animals failed to stimulate the glucose uptake of the lenses of these animals (fig. 2).

3. Plasma obtained from nephrectomized animals injected with insulin was effective in stimulating glucose uptake in keeping with our previous finding that nephrectomy did not interfere with the ability of injected insulin to stimulate glucose uptake by the lens (fig. 3).

4. Plasma from adrenalectomized animals which had not been injected with insulin significantly increased ($P < 0.001$) the glucose uptake of lenses isolated from normal animals. Insulin injection did not further increase the ability of the plasma of adrenalectomized animals to stimulate glucose uptake by lenses of normal animals. Similar results have previously been obtained when

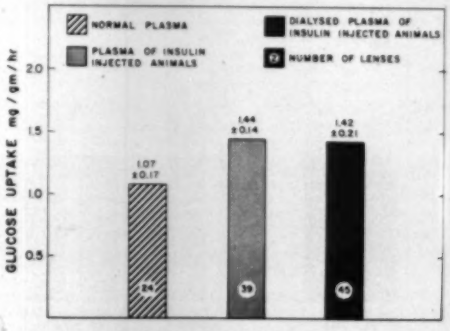

Fig. 7 (Farkas, et al.). Effect of dialysis on ability of plasma to stimulate glucose uptake of isolated rat lenses. Plasma was dialyzed against Tyrode's solution at 0°C for 18 hours then diluted 1:100 with Tyrode's solution.

lenses of adrenalectomized and adrenalectomized insulin-injected animals were incubated in Tyrode's solution containing 100 mg. percent glucose (fig. 4).

5. Increasing the concentration of plasma from insulin-injected animals increased the glucose uptake of normal lenses (fig. 5). A similar increase in effect was observed when the time interval between insulin injection and removal of the blood sample was increased (fig. 6).

6. Neither dialysis (fig. 7) nor freezing and thawing (fig. 8) altered the ability of the plasma of insulin-injected animals to stimulate glucose uptake of isolated normal lenses. On the other hand heating completely destroyed this ability (fig. 9).

Discussion

Blood plasma following the injection of insulin contains a substance which stimulates the uptake of glucose by lenses isolated from normal rats and incubated in Tyrode's solution containing glucose. This stimulatory substance is not insulin or a combination of insulin with a constituent of normal plasma, since insulin alone or the combination of insulin plus the plasma of uninjected animals will not produce a similar effect. The stimulatory substance may be a modified form of insulin or a new substance released as a result of insulin injection.

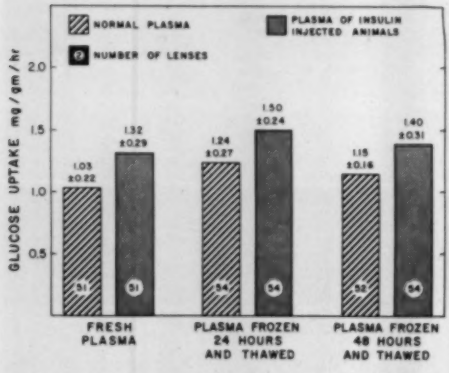

Fig. 8 (Farkas, et al.). Effect of freezing and thawing on the ability of plasma to stimulate glucose uptake of isolated rat lenses. Plasma diluted 1:100 with Tyrode's solution.

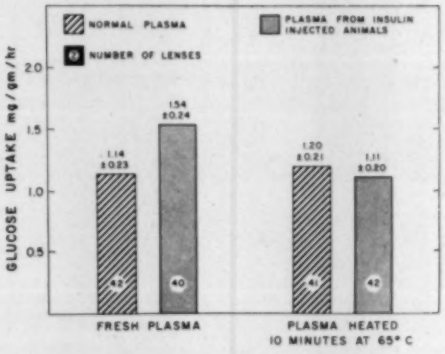

Fig. 9 (Farkas, et al.). Effect of heating on the ability of plasma to stimulate glucose uptake of isolated rat lenses. Plasma heated for 10 minutes at 65°C. and diluted 1:100 with Tyrode's solution.

This stimulatory substance is produced in a visceral organ and released into the blood. This is supported by the fact that the substance is not obtained if the viscera are removed prior to insulin injection. Sham operation and nephrectomy do not prevent the formation of the stimulatory substance. It is probable, on the basis of analogy with the results obtained with lenses from surgically treated animals,[4] that the liver is the mediating organ but this remains to be confirmed.

Adrenalectomy without the injection of insulin results in the release of a stimulatory substance and this effect is not increased further by the injection of insulin. These results are similar to those obtained with lenses obtained directly from the surgically treated animals.[4]

The stimulatory substance that is demonstrable in the blood following the injection of insulin is probably protein in nature. It is nondialyzable and heat labile. Efforts are now being made to purify this substance.

Summary

The glucose uptake of isolated normal rat lenses is stimulated when plasma from animals which have been injected with insulin is added to the incubation medium. This

stimulatory effect of the plasma is not due to the presence of residual insulin. Plasma obtained from totally eviscerated animals which have received insulin is ineffective. A stimulatory substance is present in the blood following adrenalectomy even though insulin has not been injected. These studies indicate that the stimulator substance present in the plasma of insulin injected animals may be a protein.

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Discussion

Dr. D. Venkat Reddy (Detroit): This paper is an extension of the studies previously reported by Dr. Patterson and his associates. The authors had previously shown that glucose uptake of lenses from diabetic animals was reduced. This deficiency in glucose uptake could only be reversed by systemic administration of insulin to diabetic animals prior to the removal of lenses and not by in vitro addition of the hormone to the incubation medium.

It was therefore postulated by the authors that the active principle responsible for the increased uptake of glucose was a modified form of the insulin molecule or some other humoral agent released in the body as a result of insulin action.

The investigators have succeeded in demonstrating that this is the case and that possibly the liver is responsible for bringing about this modification. They have further shown that the substance has the characteristics of a protein molecule.

Dr. John F. Kuck in our laboratories has performed somewhat similar studies though not as extensive. These data are shown in the following table: The increase in glucose uptake by the lenses incubated in media supplemented with plasma from insulin injected animals is not as pronounced as that noted by Dr. Patterson and his associates. Since there is a considerable spread in the data, the increase is not considered significant.

Insulinlike activity has been reported by several investigators to be present in normal plasma. I would like to ask Dr. Patterson if any attempt has been made to concentrate this principle from normal plasma and whether the active material in his studies is possibly identical with the aforementioned substance.

From the data reported this morning we are not able to say whether the active substance is a breakdown product or some conjugate of insulin itself or some other humoral agent. This perhaps could be established by in vitro perfusion of liver. I wonder if the authors have made an effort in this direction?

It is to be hoped that the essayists will continue these studies and be successful in establishing the exact nature of the active substance.

I greatly appreciate the opportunity of discussing this excellent paper.

Effect of plasma from insulin injected animal on glucose consumption and lactate production of ocular lens in vitro.

Glucose Consumption mg/gm/hr	
Controls	With Plasma
0.87 ± .14 (11)	1.02 ± .12 (10)
Lactate Present After Incubation mg/gm	
3.06 ± .26 (11)	3.34 ± .32 (10)

Dr. Werner K. Noell (Buffalo): Dr. Patterson's work reminded me of the work by Geiger, which is not directly related to insulin, but the artificially perfused brain fails in oxygen uptake unless the liver is in the circuit of the perfusion. Alexander Geiger in Chicago feels that the substance which delivers and which helps maintain brain oxygen consumption and glucose oxidation is uridin and cytidin. At least he found that if he gives uridin and cytidin in the artificial medium the arrangement of glucose oxidation of the brain is reversed.

Dr. Zacharias Dische (New York): I would like to point out that there is also an analogy between the findings of the Patterson group and the findings of Rein on the very specific effect of insulin on the type of oxidative processes in the

heart muscle. There, also, insulin repairs the damage to the efficiency of the oxidative processes in the heart muscle, but only if the circulation through the liver is maintained.

Apparently the liver produces some substance which is an adjuvant of the insulin.

DR. J. W. PATTERSON (closing): I would like to thank the discussers for their comments, and particularly for the points they made in regard to analogous observations regarding the activity of insulin.

There is one point which I would like to deal with briefly because I think it is well that it be in print. Dr. Reddy by his slide data obtained by Dr. Kuck in Dr. Kinsey's laboratory although supporting qualitatively the results which we presented, did have a quantitative difference, indicating an effect of 15 percent rather than an effect of 50 percent.

I want to say that we have had some difficulty for periods of time demonstrating this phenomenon, and it is quite possible that others may run into such a difficulty. The absolute reasons for it are not known.

To illustrate more specifically, this work was started originally when I was located in Cleveland; and for the period of time that we were there (and as it has been continued by Dr. Farkas, who is still located in Cleveland), the glucose uptake as indicated on our slides has followed through very nicely. However, following that, more recently I had an opportunity to establish a laboratory in Nashville; and, wishing to carry on some studies of a similar nature, we set up the necessary laboratory and animals in order to start the work.

For a period of a number of months, with a different group working, we had a very difficult time demonstrating the insulin effect. We had no difficulty demonstrating the adrenal effect, which is presumably a similar phenomenon as far as release of a stimulatory substance is concerned.

We do not know why, but perhaps changing technicians and settling down after a period of time and leaving the project for a while to work on the adrenal effect rather than the insulin effect, when we came back to it some time later we were getting exactly the same results that we were getting in Cleveland. It has been going very nicely ever since.

The only reason for mentioning this is to indicate that there are possible discouraging results that could be obtained. This does not mean that it should be abandoned. I think we need to do further work, and Dr. Kinsey and I have been talking about trying to find out just what the reason may be for this discrepancy in results under certain circumstances.

Dr. Reddy also asked about the possibility of obtaining a stimulatory substance from normal plasma. So far it has not been possible. Perhaps when the procedures are worked out for concentrating and isolating the stimulatory substance, the technique will be known whereby it might be more successfully assayed in normal plasma.

THE ENIGMA OF TYPICAL TOTAL MONOCHROMACY*

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Ann Arbor, Michigan

INTRODUCTION

Typical total monochromacy (or color blindness) is a syndrome which has classically been regarded as showing the complete absence of cone vision.[1, 18, 29, 34] However, it has recently become apparent that two kinds of functioning receptors are to be found in such eyes.[23, 24, 31, 41] The identification of the characteristics of these two kinds of receptors is, at yet, incomplete. The usual interpretation is that they are both rods.[24, 31, 41] Walls and Heath[49] suggested the possibility that they were rods and blue cones, a concept which is probably not correct in view of the discovery that seven patients of one of us (Dr. Falls) had a particular form of monochromacy, blue mono-cone monochromacy, which has some characteristics different from those of typical total monochromacy.[7, 8, 10]

RESULTS

Five typical total monochromats were examined in this study.†

* Assisted by a grant (B-1578) from the National Institute of Neurological Diseases and Blindness, United States Public Health Service.

† These studies were begun in the fall of 1955. In the intervening time two of the patients (F.B.

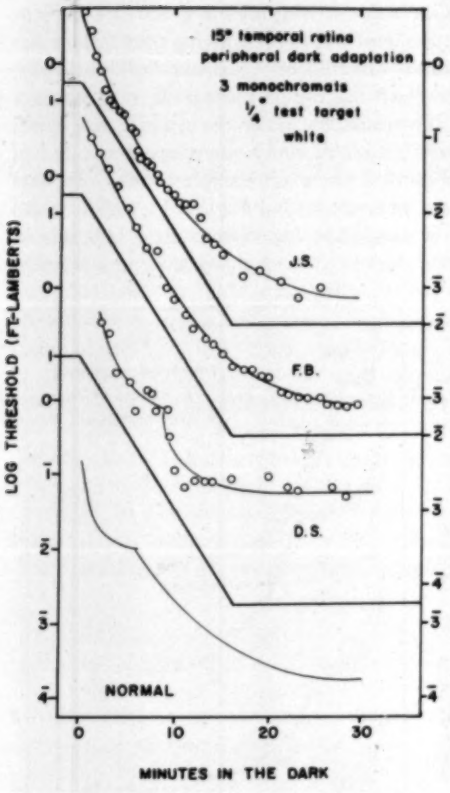

Fig. 1 (Alpern, Falls, and Lee). Dark adaptation measurements on three typical total monochromatic observers for 15° temporal retinas as compared to the normal. Ordinates: logarithm of threshold. Abscissa: minutes in the dark. These and all subsequent measurements were made on a modification of the McLaughlin[20] adaptometer. The subject was dark adapted for 15 minutes then light adapted for 10 minutes prior to the measurements. The adaptation light in every case but one was 1700 ft-L. In the case of F.B. it was 3000 ft-L. Natural pupils were used.

1. *Deficiency in cone function.* Figure 1 shows measurements of dark adaptation function in the 15 degree temporal retina on the three patients given this test. In two of the three cases there is a single monotonic dark adaptation curve extending over four logarithmic units. The normal dark adaptation curve is clearly divisible into two parts, respectively due to cones and to rods.[22] The single curve in the cases of typical monochromacy suggests strongly that these eyes, in the region of the retina tested, have few if any effective cones.* Similar data have been obtained by others.[24, 39, 49]

A second reason for suspecting a deficiency of cone function in typical monochromacy is that there may frequently be a clear reduction in the ability of a given accommodation stimulus to evoke an accommodation response in these eyes (Fig. 2). It does not suffice to say that this impairment is due merely to the reduced visual acuity in such eyes, as Heath[19] has proposed, because: (a) normal eyes with similar reduced visual acuity produced, say, by reduced light level will show much more prominent responses in this test[2] and (b) the same result is obtained with the eyes of monochromatic observers even when the test letters are made sufficiently clear (by enhanced contrast and increased letter size) as to be quite legible to them. Recent work has emphasized the importance of proper cone vision for the normal functioning of the accommodation reflex.[12, 15]

2. *Double receptor system.* There are, however, two distinct types of receptors, one of which operates at low levels of luminance, the other at high. This is the easiest to see in Figure 3 which shows the dark adaptation curves for the center of the visual fields of five monochromats.

While the normal eye showed in this condition only a single smooth monotonic curve (since the center of the fovea contains no rods) the monochromatic eyes all showed a double function. In the most reliable cases the final threshold was somewhat below the normal fovea threshold. In the younger subjects they were somewhat more elevated than

and L.B.) were also studied to some extent by O. M. and H. R. Blackwell,[19] while members of this department.

* This is not an invariable finding however since in one of the three cases studied and in several others described in the literature[41, 49] a double dark adaptation curve is sometimes found in the peripheral field.

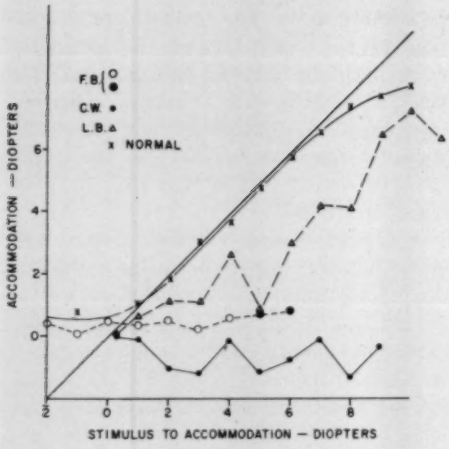

Fig. 2 (Alpern, Falls, and Lee). Accommodation response to the gamut of accommodation stimuli on three monochromatic eyes. All measurements were taken with only one eye, but fixation in each case was binocular. The data for F.B. were obtained by variation in the magnitude of the stimulus to accommodation by addition of concave and convex lenses while the patient fixated a chart at 340 cm. from his eye. With the open circles the letter size was equivalent to 6/27.4 with the closed circles it was 6/105.5. The data for C.W. and L.B. were obtained by moving the chart closer and closer to the eyes. In all cases the subject was able to read the letters which were being fixated. Points are means of three independent measurements made with the method of stigmatoscopy.[3] Natural pupils were used. The data for L.B. have been arbitrarily elevated 1.55 diopters; those for C.W. approximately 1.40 diopters. No very large amount of "artificial myopia" such as was reported by Heath[19] was found in these cases.

this. The double dark adaptation curve has been reported for the periphery[31] and for both the fovea and periphery[41,49] of some monochromats. It strongly suggests the presence of two kinds of photoreceptors. If one can rely on the precision of fixation, the data are evidence also that the .25 degrees center of the fovea in these eyes also contains rods. Further evidence of this conclusion has been obtained in the failure of two of our observers to have a central scotoma for very dim lights. This latter, however, is not an invariable finding.[26]

It is useful to study other conditions in which suggestions of a duplex retina appear. One way of doing this is to observe the amplitude of nystagmus during dark adaptation in the two subjects (F.B. and L.B.) in whom a clear horizontal nystagmus was evident. The measurements on the eye of F.B. (which are typical in every way) are illustrated in Figure 3 (in which samples of the raw data are presented) and Figure 4 (which shows the amplitude for various time intervals in the dark). The result of this measurement

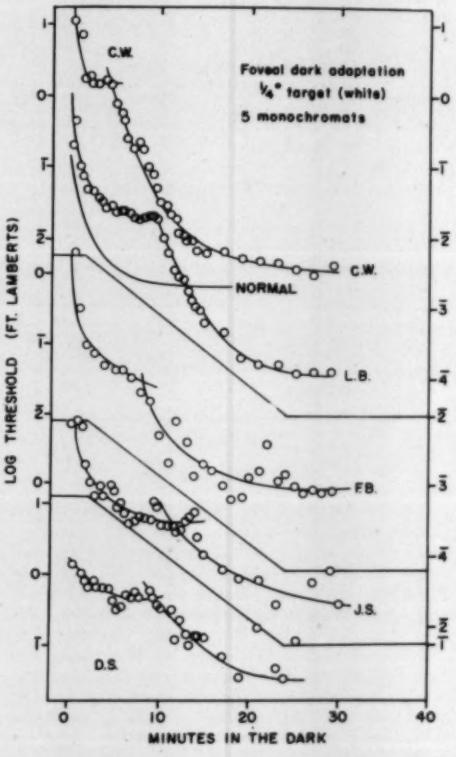

Fig. 3 (Alpern, Falls, and Lee). Foveal dark adaptation measurements on all five subjects and on the normal eye. The apparatus, adaptation procedure, and method of plotting are the same as that described for Figure 1. The fixation target was a pattern of 4 tiny light spots arranged in diamond fashion around the point where the test flash (¼°) appeared. The distance between the horizontal light spots was 1¾° (and this was exactly that between the two vertical light spots as well). Natural pupils were used but the data for C.W. were obtained with the pupil artificially dilated with a mydriatic.

revealed a fine pendular conjugate nystagmus of about four degrees. This was greatest in the light but began to decrease in amplitude very soon after entry into the dark and this nystagmus persisted during the early dark adaptation periods. As soon as the second ("rod") part of the dark adaptation curve began, (c.f. Fig. 9) the nystagmus was completely gone. According to the pure rod hypothesis one would anticipate that the nystagmus must persist even under scotopic conditions as Walls and Heath have already pointed out.[49] Clearly this is not the case.

Still further evidence for the duplex character of the typical monochromatic retina is illustrated by measurement of the consensual photo pupil response (Fig. 6). Both rods and cones contribute to the photo pupil response in the normal eye.[3] Furthermore the relation between pupil size and retinal illuminance in the normal eye shows a duplex

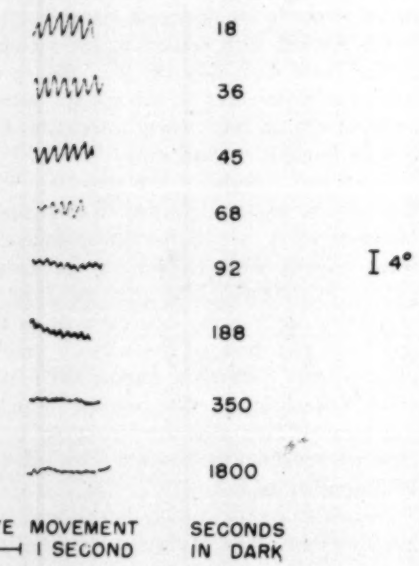

Fig. 4 (Alpern, Falls, and Lee). Raw record of the nystagmus (F.B.) for various indicated intervals following light adaptation (16° field in Maxwellian view) to $2\cdot(10)^5$ trolands for 5 minutes. Measurements were made by electro-oculography. The subject fixated a small point of light in an otherwise dark room.

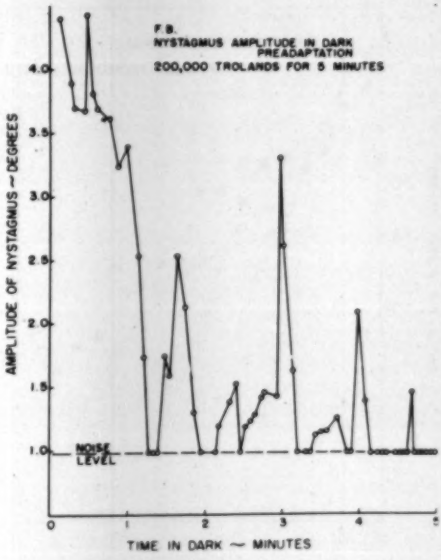

Fig. 5 (Alpern, Falls, and Lee). Variation in the amplitude of the nystagmus at various time intervals in the dark (F.B.). Ordinate: amplitude of the movement, abscissa: minutes in the dark.

curve.[16] According to the usual interpretation of such curves[22] one may suspect that the low level curve represents action of rods, the high level curve action of cones in the normal eye. In Figure 6 the low level curve of the monochromatic eye seems to show that at these intensities the pupil is, if anything, more sensitive to light than is the case in the normal eye. At high levels quite the reverse seems to be the case. Clearly a double curve is necessary to describe the photopupil intensity relation in this eye, just as it is in the normal eye.*

* We have been unable to verify the observation of Geldard[18] who found that "... only a very minute and immeasurable twitch ..." of the pupil could be evoked by a light stimulus. Nor do we duplicate Engelking[14] who found rigid pupils in light adaptation but prompt responses after dark adaptation. Our results are exactly in agreement with Hess[27] who found prompt responses both in the light and in the dark adapted eye, in typical total monochromatism. In the literature the typical total monochromat is frequently described as having a "sluggish" photo-pupil response. While the precise interpretation of the term is not clear, the data of

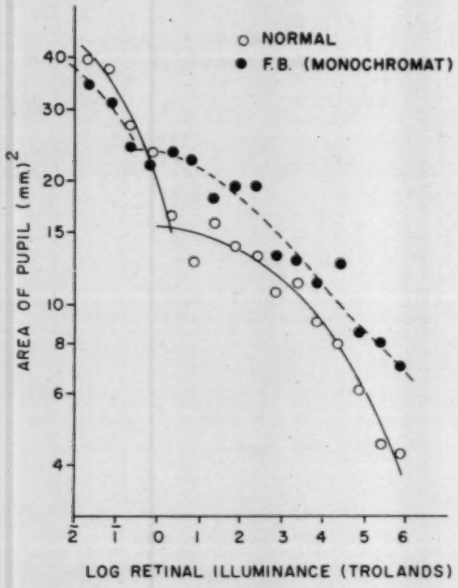

Fig. 6 (Alpern, Falls, and Lee). Area of the pupil for the normal eye and for F.B. as a function of log of the retinal illuminance in trolands. The measurements were obtained by infrared photography of the left eye while the right eye was exposed to a steady state Maxwellian view of 13.5°. The observer was first dark adapted for 30 minutes and then exposed to a field of lowest luminance level. After adapting to this level, three photographs were taken. The light level was increased by one-half logarithmic step, the observer adapted to this new level, and the process was repeated. In this way measurements were made going from the lowest to the highest level. The plotted points are the mean data.

3. *Spectral analysis.* Measurements were made on a Hilger constant deviation prism monochromator of the spectral sensitivity of the center of the visual field of all five of these observers.* The results of these measurements are illustrated in Figure 7 which shows the radiance at each wave length required to establish a brightness match with a standard comparison surround at 1.85 trolands of retinal illuminance. The mean data of the measurements on four normal observers are included in this figure for comparison. The most striking feature of the data is the greatly reduced sensitivity of the monochromat at the red end of the spectrum compared to the normal eye. The latter curve has a minimum at about 560 mμ under these observation conditions. All of the monochromats have minima considerably less than this value. In fact the data for each of the monochromats are closely approximated by the C.I.E. (1951) scotopic curve although in only one case (L.B.) is the agreement reasonably exact. In the two youngest adolescents (J.S. and D.S.) the deviations seem to be more or less random suggesting errors of measurement, while in the other cases the curve seems to be displaced somewhat towards the red with respect to the scotopic curve. These data show the same degree of individual differences in the spectral sensitivity of typical total monochromatism that is to be found in the literature.[1, 6, 24, 28, 31, 45]

Individual differences are important and they will be discussed further in this paper. However, they are rather inconsequential when viewed with respect to the rather prominent differences between the spectral sensitivity curve of the monochromat on the one hand and those of the normals on the other. Figure 8 shows a comparison of the mean monochromatic data and the mean of the normal data. The curves agree quite closely throughout the blue but show marked deviations in the red.

As a first approximation the spectral sensitivity of the center of the visual field of the

Figure 6 do show that for the high intensity curve the rate of change of pupil area with intensity is usually somewhat less than is the case for the normal eye. It is possible that one may anticipate much wider individual difference among monochromats in this characteristic than in some of the other ones.

* The modifications of the monochromator necessary to adapt it for this purpose were designed and the instrument calibrated by Mr. B. S. Pritchard under the direction of Prof. Blackwell when he was a member of this department. The detail description of the apparatus, its calibration and the method of making these measurements and a variety of data on normal eyes are given in a paper by Prof. Blackwell[9].

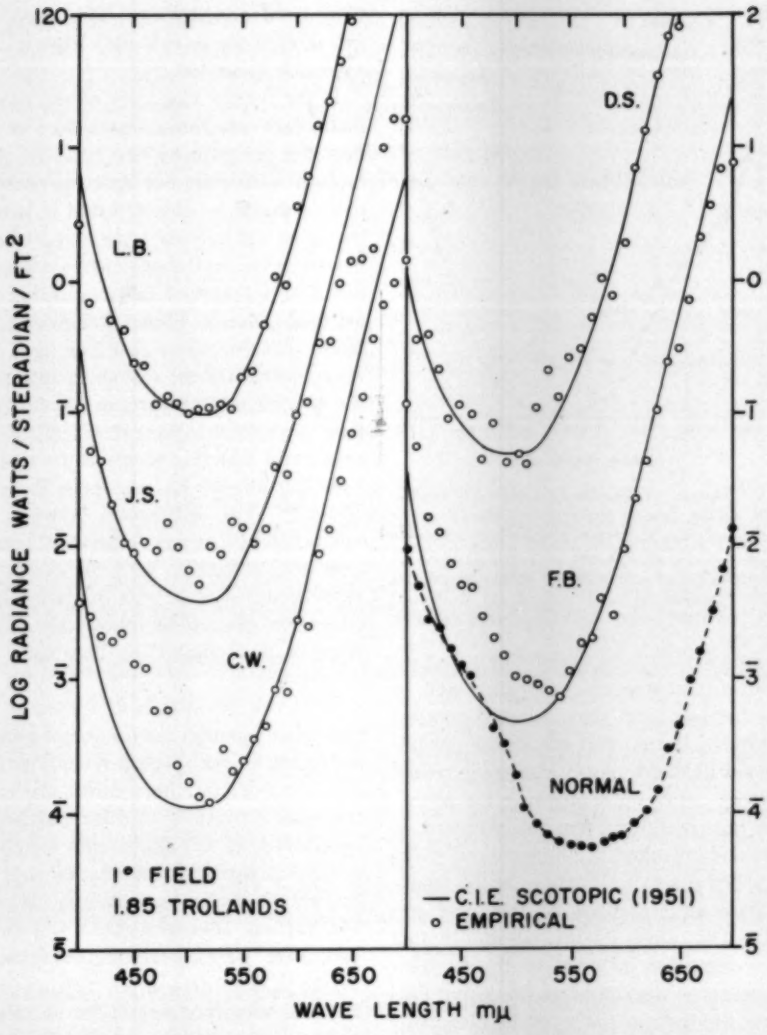

Fig. 7 (Alpern, Falls, and Lee). Radiance at various wave lengths throughout the spectrum required to match 1.85 trolands of retinal illuminance using the method of cascade for five monochromats and for (the geometrical mean of) four normal eyes. The subject was instructed to fixate the center of a 20' monochromatic test field which was surrounded by an annulus with 1° outside diameter. This annulus was made monochromatic by narrow band interference filters and the subject made a brightness match between the center and the surround. Large differences in chromaticity were avoided by frequent changes of the interference filters. Each repetition consisted of cascade up and down through the spectrum. The plotted points are empirical. The smooth curve is the reciprocal of the C.I.E. 1951 scotopic spectral sensitivity curve. The data for the lowest pair of curves occupies the correct position on the graph. The intermediate pair of curves have been arbitrarily shifted up one, the upper pair two, logarithmic units for the sake of clarity. All the data are only a single repetition except for F.B. for whom the experiment was repeated twice.

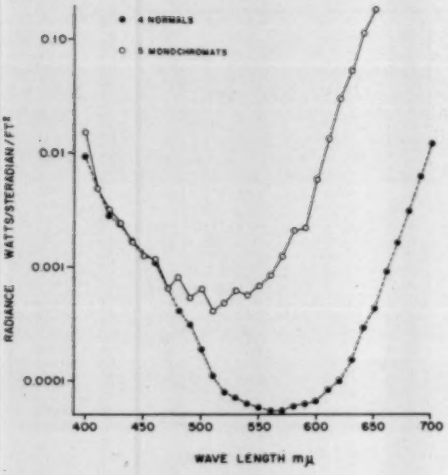

Fig. 8 (Alpern, Falls, and Lee). Geometric means of all the data of the radiance at each wave length through the spectrum required to establish a brightness match with a test field of 1.85 trolands using the method of cascade for four normal and five monochromatic eyes. The data for F.B. and J.S. were repeated twice. All others were repeated a single time.

monochromat compares reasonably well to that of the low level dark adapted spectral sensitivity of the normal eye in the peripheral visual field. Moreover changing retinal illuminance either up or down in the identical viewing condition from the standard of 1.85 trolands resulted in no change in shape of the spectral sensitivity curve in the monochromat at all regardless of the level employed.* This suggests the possibility that the photosensitive material in the receptor which subserve the center of the visual field in these monochromatic eyes is essentially that which subserves night vision in the peripheral retina of the normal eye. Before considering this possibility, however, one important factor must be taken into consideration. It is possible that the measurements just described were carried out on a somewhat peripheral region of the retina while the patient fixed eccentrically. This possibility is not an easy one to rule out.† Thus an indirect approach is needed.

If the duplex character of the monochromatic dark adaptation curve does in fact reflect the presence of two types of photoreceptors with different spectral sensitivities, then it would be expected that if two different color test lights are employed to measure dark adaptation, the two curves will not have identical form even when all other factors are held constant. Figure 9 shows dark adaptation data for white and blue light, the latter corrected for the scotopic transmission of the 47B filter. Both parts of the dark adaptation curve seem to have the identical spectral sensitivity and this seems to be that of the rods. This result has also been described by Sloan.[41a] The difference between typical monochromats in this respect and blue monocone monochromats is quite sharp. In this latter case the early part of dark adaptation curve for white light follows the blue curve when it is corrected for blue cone spectral sensitivity.

If it can be imagined, for some reason, that the adaptation curves do not give a valid indication of the spectral sensitivity of these high intensity receptors either, one can make the same analysis with other psychophysical measurements. Measurements were made of acuity, intensity discrimination and critical flicker frequency as functions of intensity for various colored lights. The results in each case were precisely the same. These curves differentiate into a high intensity and low intensity section and the spectral sensitivity of each section is essentially that of the

* Curiously enough measurements in the *normal* eye do show rather marked differences as has recently been documented by Blackwell and Blackwell[9].

† If the fixation is eccentric, however, it is so by only an extremely small amount. Hess and Hering[28] showed that the blind spots of their patient were in exactly the same position in the visual field as those of the foveally fixating normal observer. This fact was verified completely in the only two present cases (F.B. and C.W.) in whom it was tested. Furthermore the fact that the size (as well as the position) of the blind spot in subject F.B. was exactly the same as that found in normal controls suggests the possibility that he makes all his observations when the eyes are exactly at the same position in the nystagmus swing.

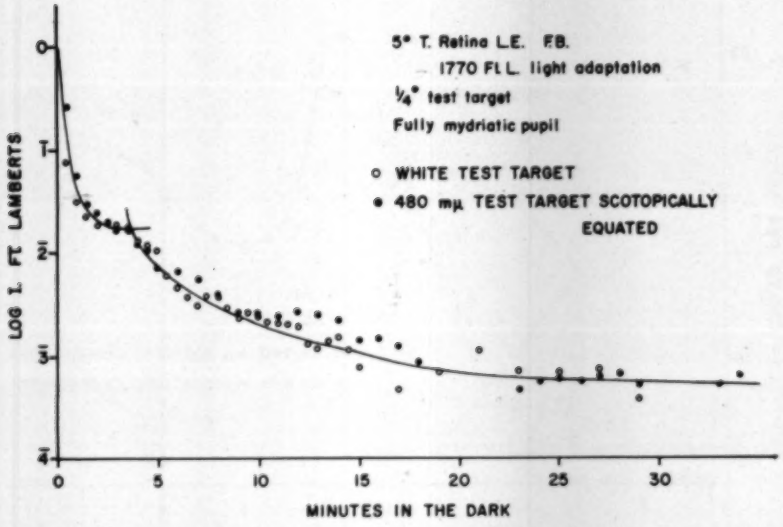

Fig. 9 (Alpern, Falls, and Lee). Dark adaptation curve for a 5° temporal retina area of the left eye of F.B. following 3 min. light adaptation to 1770 ft.-L for a white (open circles) and a blue (solid circles) test target. When the data for the latter are equated for the scotopic transmission of the blue filter, the two curves more or less exactly coincide within the limits of measurement error. Pupil fully dilated and made unresponsive to light by topical application of a mydriatic.

rods.[24] Figure 10 illustrates the data obtained on F.B. for red, white and blue flickering stimuli.*

4. *Directional sensitivity.* The question remains to be answered as to whether these high intensity receptors in the color blind retina have the anatomical characteristics of rods or of cones. The traditional view maintains that they are rods. Walls and Heath[49] believe they are cones. The eyes of the only monochromatic observer ever to be given a histologic examination had normal cones in the retina except for the fovea. In the fovea the cones were larger in diameter and had outer segments of very small size (if they had any at all).[30†] No additional histologic data accompanies the present report. However one can take advantage of the fact that the tapered sides of human cones are probably related to their high degree of directional sensitivity.[33] Rods normally have no such directional sensitivity.[17] Do the high intensity photoreceptors of monochromatic eyes which have just been shown to have spectral sensi-

* The exact form of the curve above 100 trolands is difficult to explain. All of the psychophysical measurements at these high light levels show a shift in performance in the direction of the data obtained at much lower intensities. This may be related to influences of the surround since the size of the surround was only 6°. For normal eyes at very high light levels very small surrounds produce a decrement of c.f.f.[20, 28] but this decrement can be eliminated by increasing the surround to 35°.[21] It is possible that whatever factors in the normal eye operate to reduce the c.f.f. after it has reached its maximum (when the surround is small) are somewhat exaggerated and come into play at a lower intensity in these color blind eyes. Difficulty in seeing at very high light levels is one of the characteristic features of typical total monochromatism.

† Walls and Heath state that ". . . one can gather that Larson's micro-technique was impeccable." However, Rushton has called our attention to the fact that the actual report states with regard to the right eye ". . . Leidier war nicht genügend Solutio formaldehydi in diesen Bulbus getraufelt worden, so dass sich ein bedeutender Verfall der Stäbchen-und Zapfenschicht zeigte. . . ." The work on the other eye apparently was free from such artifacts and there seems to be no valid reason for denying the observations which were made on it.

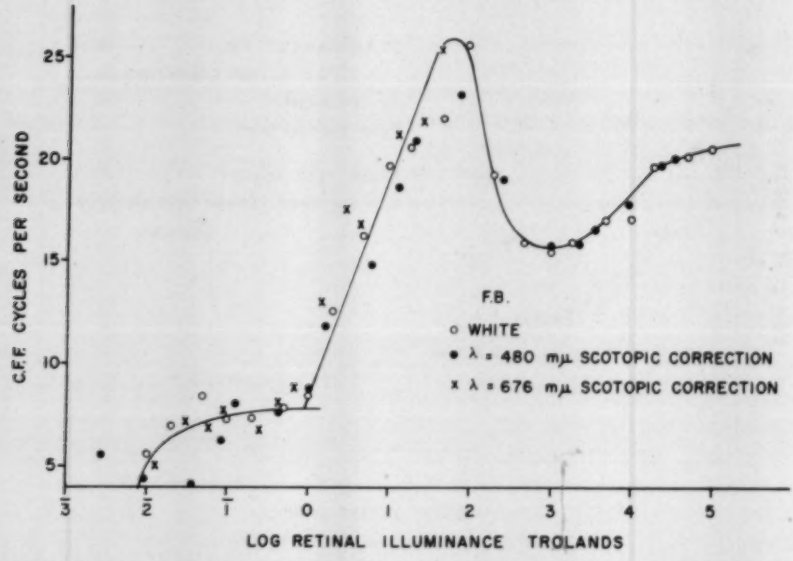

Fig. 10 (Alpern, Falls, and Lee). Critical flicker frequency as a function of retinal illuminance for white, blue and red for the center of the field of monochromatic observer F.B. The flickering field was 2° in diameter with an annulus surround of 6° outside diameter. The apparatus has been described in a previous paper.[4] The adaptation precautions used in the pupillographic experiment were also repeated here. The artificial pupil was 1.4 mm diameter. The data for the colored lights are corrected for the scotopic transmission of the filters. The illuminance of the surrounds was equated to the talbot illuminance of the flickering stimulus as long as this latter was equal to or less than 100 trolands. For all values higher than this, the illuminance of the surrounds was held constant at 100 trolands.

tivities closely resembling that of rods have the directional sensitivity of cones?

To answer this question the observer was positioned with head fixed rigidly in an apparatus which could be moved in a horizontal direction in a plane normal to the optical axis of the apparatus used for measuring critical flicker frequency.[4] In this way, it was possible to transverse the subject's pupil measuring c.f.f. at a fixed intensity for various values of eccentricity (r) from the nasal edge of the pupil to the temporal edge (and then vice versa) and thus to measure the directional sensitivity of the total color blind retina (that is, its Stiles-Crawford Effect). In the present case, measurements of directional sensitivity were made on four of the five monochromats.* Unfortunately, owing to the limited available time, only preliminary measurements could be made in three of the four cases. These data, however, were sufficient to verify the fact that in each of these eyes a clear directional sensitivity of these high intensity photoreceptors existed. In one case (F.B.) a more detailed series of measurements were possible. In this case the in-

* Light entering different parts of the pupil even though incident on the same retinal regions, does not have the same effect on brightness.[6] The method used in this work to measure the Stiles-Crawford Effect has not, in so far as we know, previously been used to do so. The more usual method of making such measurements is by establishing a brightness match between two photometric surfaces one of which is seen by light reaching the retina through the center of the pupil, the other by light reaching the retina through various eccentric positions (r) within the pupil. The measurements using the flicker method on normal eyes however give results quite comparable to those obtained by Stiles and Crawford. The flicker method offers a number of advantages in working with clinical material not the least of which is its simplicity and the ease with which naive patients give valid observations.

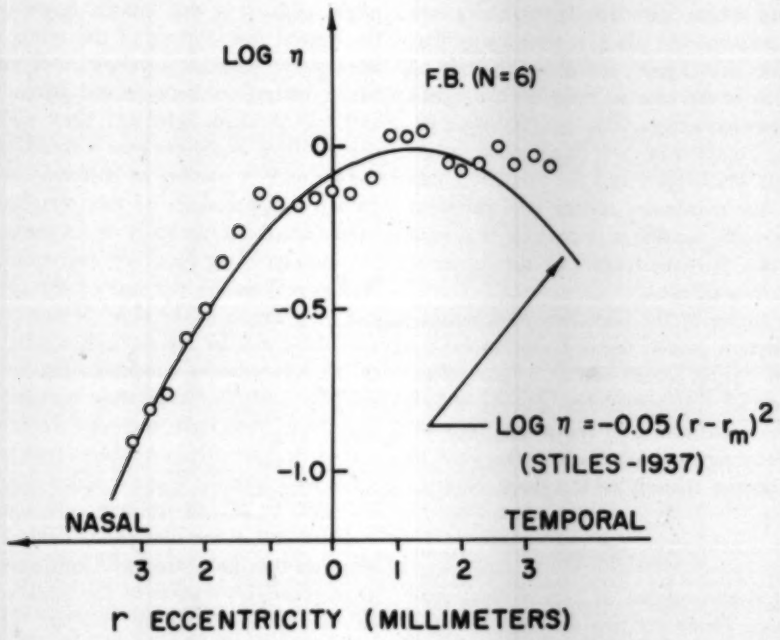

Fig. 11 (Alpern, Falls, and Lee). Directional sensitivity measurements of F.B. Ordinate: the difference in the logarithms of the retinal illuminance (for central entry) required to produce the c.f.f. obtained at the peak value r_m and that obtained at point r. Abscissa: eccentricity r of the pupil in mm. Apparatus was the same as that used to obtain the data in Figure 10 although a rigid head clamp with dental bite was added. This permitted the entrance pupil of the eyes to be moved in a plane normal to the optical axis of the flicker apparatus in very small but calibrated steps. The points are the means of six measurements on the eye of F.B. The curve is drawn from the equation suggested by Stiles[42] to fit the data of his own eye.

tensity of the flickering stimulus was set at a value such that the c.f.f. (for central entry) was in the region of the high intensity part of Figure 10, in which the relation between c.f.f. and logarithm of retinal illuminance was linear. The relative luminous efficiency (η_r), of each point of pupillary entry r could be computed by determining the ratio of retinal illuminance required (for central entry) to give the c.f.f. obtained at the point r to that of the retinal illuminance required (for central entry) to give the maximum c.f.f. obtained. The results of the measurements when analyzed in this way are illustrated in Figure 11, in which the values of the log η_r are plotted as a function of point of pupillary entry r. The plotted points are the mean of the six measurements, the curve is the empirical equation found by Stiles[43] to fit the data for his own eye. The agreement between the empirical data and the curve in Figure 11 emphasizes the fact that the photoreceptors which subserve flicker discrimination at high intensities in the eye of F.B. have a directional sensitivity quite like that found in normal cones. It has already been pointed out, however, that these identical photoreceptors have a spectral sensitivity quite similar to normal rods.

One other feature of the data in Figure 11 must be pointed out. This is the fact that the maximum visual effectiveness in the eye of F.B. occurs not for light entering the center of the pupil but for light entering 1.25 mm. temporal to this point. The most logical interpretation of this result is that the photo-

receptors whose directional sensitivity are being measured are tilted in such a way that their axes are aligned, not along the line of sight, (as is the case in most normal eyes) but somewhat eccentric to it. The same result was obtained in each of the four monochromats who were tested for this effect (although the maximum occurs at a different point in each) so that it is possible that such tilting is a constant feature of the retina of monochromatic eyes.

This tilting of the photoreceptors may be an important part of the explanation for the reduced central visual acuity in monochromatic eyes.* Campbell[13] has already shown that the visual acuity of the normal eye for rays entering the pupil obliquely may be much poorer than it is for those entering centrally.

Discussion

This is the enigma of typical total color blindness. There are two different kinds of receptors, one of which functions at low intensities, the other functions at high intensities. The former is undoubtedly similar to normal retinal rods, the latter has the directional sensitivity (and presumably the same anatomic characteristics) of normal retinal cones but the spectral sensitivity quite similar to that of normal retinal rods.

One can propose at least two reasonable explanations for these facts but neither is completely satisfactory.

(a) It is possible that the high intensity photoreceptors are perfectly normal cones except for the presence of rhodopsin within them rather than the normal cone pigment (or pigments). If this were true, the spectral sensitivity of these cones would be identical to that of the C.I.E. scotopic spectral sensitivity curve. The data in Figure 7 show good agreement with this but individual differences can be observed and in only one case (L.B.) is the agreement as good as one might wish. It is well known, however, that the central five degrees of the retina in the normal eye contains a yellow filter material which intervenes between the foveal cones and the incident light and thus influences chromaticity of objects seen with this part of the retina.[46] A number of objective and subjective measurements of this material have been carried out, the most recent are those of Rushton on living eyes. This pigment is quite transparent at the red end of the spectrum but very dense in the blue. If one assumes that the cones in the monochromatic retina do in fact contain rhodopsin but that this macular pigment intervenes between them and the incident light, then one would expect the spectral sensitivity of these cones to agree quite well with the scotopic spectral sensitivity curve at the red end but to be somewhat reduced in the blue. The present data (fig. 7) suggests this quite strongly. They agree with various similar reports in the literature, one of the earlier being by Hering[25] and one more recently by Hecht, et al.[24] The possibility of the disparity between Hering's measurements in his own dark adapted eye and those of his monochromat's fovea might be due to macular pigment absorption was pointed out long ago.[40] Hecht, et al.[24] suggested this explanation also for a similar discrepancy in their case. In order to quantify this, one may vertically displace the C.I.E. scotopic curve until it coincides precisely with the measurements from the monochromatic retina in the red end of the spectrum. By then deducting the logarithm of the relative spectral sensitivity of the monochromatic eye from the logarithm of the ordinate of this displaced curve, one obtains a direct measurement of the optical density of the inferred macular pigment.

Figure 12 shows optical density of this inferred pigment as a function of wave number (reciprocal of the wave length) as determined from the mean spectral sensitivity curve of the five monochromatic observers for the center of their visual fields (if not for the center of their foveas).*

* If this is true one might expect poorer acuity with a grating test object normal to the meridian of tilt than with a grating parallel to this meridian.

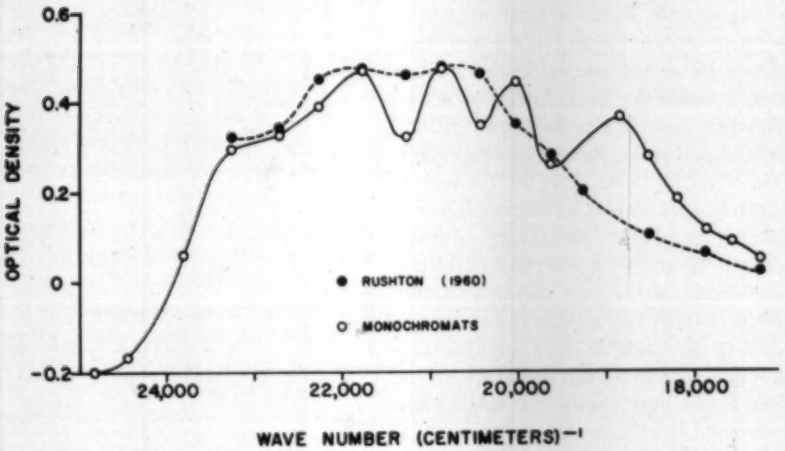

Fig. 12 (Alpern, Falls, and Lee). The inferred macular pigment for (the geometric mean of) the five monochromatic eyes compared to the *in vivo* measurements of Rushton.[36] Ordinate: optical density; abscissa: wave number (centimeters^{-1}). The curves are neither shifted nor arbitrarily equated.

Also illustrated in this figure are Rushton's[36] unpublished objective measurements† of in vivo macular pigments of normal eyes obtained by the method of reflection densitometry. The agreement is probably reasonable enough to suggest that macular pigment may play an important role in the discrepancies illustrated in Figure 7. While there is a fair agreement of the two sets of data in Figure 12, it is not as exact as one might hope. What is the reason for the differences in the two sets of curves? While measurement errors certainly have to be considered, it should also be pointed out that the product of visual purple bleaching is a colored substance. In the moderately high light adaptation levels of the foveal luminosity measurements of Figure 7 this substance might well alter the spectral sensitivity curve obtained. This would be less so in the case of the C.I.E. scotopic curve which is based on absolute threshold measurements (and thus considerably smaller amounts of bleaching). The possibility, however, that the fovea of the monochromatic retina contains a small amount of additional pigment which is related neither to rhodopsin (and its bleaching products) nor to the macular pigment of the normal fovea, measured by reflection densitometry, cannot be conclusively ruled out, with the data so far available.

If both the cones and the rods of the monochromatic retina contain rhodopsin then

* This curve is drawn from the geometric mean of all the measurements of the monochromatic eyes made at 1.85 trolands. There is good evidence, however, of rather marked individual differences in the presence of this inferred pigment and this is evidence by the differences in the extent to which the various curves in Figure 7 fail to agree with the scotopic C.I.E. curve. Thus the data for L.B. agree so well with the latter that it is hard to imagine there is any additional pigment at all in the region of his retina with which these observation were obtained. On the other hand, the data from C.W. and F.B. suggest the presence of a rather prominent amount of the inferred pigment. Apparently normal eyes, too, show rather large individual differences in the amount of macular pigment to be found in them.[46] According to this theoretic position the differences in the position of the maxima of the spectral sensitivity curves of the center of the field in the various typical total monochromatic eyes described in the literature and verified in the present study would be on the basis of the individual differences in the amount (and perhaps also in the absorption spectrum of) the macular pigment.

† Rushton's data show greater absorption toward the red end of the spectrum than xanthophile which is what is commonly regarded to be the identity of the macular pigment.[46] They agree reasonably well with Wald's subjective estimates, however, and certainly are the most precise objective data on living eyes so far available.

clearly the double dark adaptation curve (found with measurements on either the monochromatic or the normal eye) can not be due to a difference in the identity of the photosensitive materials in these two different kinds of receptors. Recent evidence suggests the possibility that receptor-for-receptor rods and cones may indeed have the same absolute sensitivity.[5, 50] According to this view, the differences in the final absolute threshold value of the two plateaus of the dark adaptation curve is linked not to the difference in the kinds of the respective pigments in rods and cones but rather to differences in spatial and temporal summation characteristics. Evidence for influences of effects of this kind on dark adaptation has been emphasized recently by Rushton.[37] However Rushton[39] has also shown in his case of total color blindness that the shape of the dark adaptation curve (logarithm of threshold versus time) in the cone free retinal region exactly follows the regeneration curve of rhodopsin (as the former declines over seven logarithmic units) after total bleaching. In this case he was able to show a linear relation between the log of the threshold and the fraction of rhodopsin bleached as predicted by Wald's[47] compartment hypothesis.*

b. A second possibility is that the cones of the monochromatic fovea are completely normal cones containing whatever pigment similar cones in the normal fovea do. If this were true then one would explain the differences in the vision of these as compared to normal eyes by the absence of certain types of cones in the monochromat's retina. One can then get an idea of what the spectral sensitivity of these missing cones would be like by obtaining a difference spectrum between the two sets of curves in Figure 8. The results of this calculation are shown as the open circles in Figure 13. In the same figure the solid circles illustrate the absorption spectrum of a photosensitive pigment measured by Rushton[36] in the normal fovea and which he later called erythrolabe.

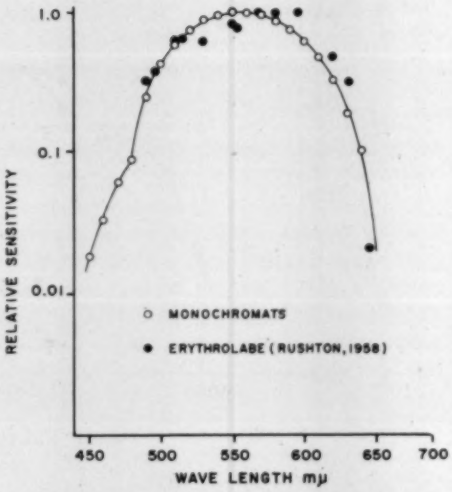

Fig. 13 (Alpern, Falls, and Lee). The spectral sensitivity of the inferred pigment presumably lacking from the monochromatic "fovea" (geometric mean of 5 observers) and the measurements of Rushton[36] of erythrolabe in the normal fovea. To obtain the data represented by the open circles the data in Figure 8 were converted to a quantum basis and the reciprocals of all values were then made proportional to the value of the normal eye at $\lambda = 560$ mu. Smooth spectral sensitivity curves were then drawn through each set of curves and the difference between the ordinates at each wavelength represented the "sensitivity" of the photoreceptor (or photoreceptors) presumed to be missing from the monochromatic fovea. These data are plotted on a relative basis in this figure.

The agreement between the two very different kinds of data is reasonable enough but the hypothesis leaves much that is not explained. Are the cones which remain in the monochromat fovea of a single type or a mixture of two or more types? On the one hand if they are but a single type then they obviously must be green cones since the characteristics of these observers are different from those of the blue mono-cone monochromats. But the spectral sensitivity of the monochromats cones differs quite markedly

* Actually a number of difficulties with the compartment hypothesis have been pointed out by Rushton himself[37] and it seems quite probable that another explanation for Rushton's result will be found.

from what other kinds of evidence suggests the green photosensitive material to be.[36, 44] It is true that the cones of the fovea of these color blind eyes have a rather marked degree of tilt. Furthermore the color vision of an object differs accordingly as the light from the object strikes the cones at different angles of incidence.[11, 43, 44] The discrepancies are in the right direction to account for the results, but a quantitative verification must await much more adequate information about this effect in normal eyes. On the other hand, if the cones in the fovea of the total color blind eye are a mixture of several different types, then how is the absence of color vision to be explained?

This then is the enigma of typical total color blindness. A more complete understanding of it must await much more laboratory data. Curiously enough, such information may come, not from more exact measurements on the total color blind eye (although certainly much more needs to be done here), but from data on normal eyes.

Summary

1. Typical total color blind eyes show evidence (from dark adaptation studies and studies of the accommodation mechanism) of being deficient in the number of functioning cones.

2. Such observers however show evidence of two different kinds of photoreceptors, the first of these functions at low levels of illumination and is probably identical to normal retinal rods. The second of these functions at high levels of illumination and has many characteristics of normal retinal cones.

3. The directional sensitivity of the high intensity photoreceptors in the typical total color blind retina is essentially that of the cones of the normal eye. These monochromatic cones, however, seem to have an extreme degree of tilt.

4. The spectral sensitivity of these high intensity photoreceptors is very similar to that of the normal retinal rods.

5. These facts may be explained by two alternate hypotheses: (a) that the monochro-

APPENDIX

Summary of Data

Patient	Age	Sex	Farnsworth-Munsell 100-hue Test	Corrected Far	V.A. Near	*Macula*	Arago Phenomenon	Family History
J.S.	11	M	616 errors	20/200	J13	R. Normal pigmentation smooth texture L. Inactive central chorioretinitis	No	Brother to D.S., older brother also similarly affected.
D.S.	14	M	655 errors	20/200	J13	Marked granularity of pigment in macular area more on R than on L	Not tested	Same as J.S.
L.B.	17	M	645 errors	R 4/60 L 6/60	J7 J7	Minute pigment stippling. No gross clumping	Yes	Two brothers with similar defect.
C.W.	14	F	1,046 errors	R 6/60 L 3/60		Minute foveal reflex. Macula area normal	Yes	None.
F.B.	30	M	953 errors	R 20/200 L 20/80	J7 J1	R. No foveal reflex; no pigment degeneration; no clumping L. Slight pigment degeneration and clumping situs inversus O.U.	No	Father reported similarly affected. Was not seen.

matic cones contain rhodopsin or, (b) that the monochromatic cones are some of the cones of the normal retina at a rather large degree of tilt.

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We would like to acknowledge the kind assistance of each of the following: Mr. Benjamin Pritchard who designed and calibrated the modifications of the Hilger prism monochromator for the measurements of luminosity; H. Richard and O. M. Blackwell whose wide experience with this instrument was of considerable assistance in a number of ways in its application to these observers; W. A. H. Rushton, F.R.S., who made many very helpful suggestions including that of the experiments on directional sensitivity and also generously permitted us to use his unpublished measurements of the macular pigment in the normal retina illustrated in Figure 12; and D .W. McCready, Jr. who assisted with the pupillographic data.

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Discussion

Dr. W. A. H. Rushton: In normal eyes there are three classes of ways in which we can distinguish whether rods or cones are functioning. There is the evidence from the structure, the evidence from the chemistry of the visual pigments, and the evidence from the nerve organization—such things as acuity, flicker, and so on.

When we have eyes as abnormal as those of the rod monochromat, we are not secure in getting evidence from anything except structure. We have no grounds for supposing that either pigments or nerve organization are at all normal. As you heard, the structure obtained from histology is almost nonexistent.

Dr. Gordon Walls, whom you all will be disappointed not to see dash into this fray as indicated on the program, I seem to remember has written that he has obtained data from a laboratory porter with this condition, and I am sure Dr. Walls intends to outlive him. But until that or similar circumstances take place, we haven't got the histology, and that is where this communication becomes so extremely important, because it does demonstrate for the first time—and indeed, better

than histology could—that the high-level receptors are in fact cones.

Flamant and Stiles in man, and Donner and I in the frog, have shown that the Stiles-Crawford effect does not occur with rods; but Alpern and Falls have shown that it does with these high-level receptors. So, the high-level receptors just cannot be rods. There is no example known when the Stiles-Crawford effect (in the way they showed it) can occur with the rods, but all the examples show that it occurs with the cones if they are just a little displaced.

So, that evidence is absolutely convincing, and it is the first piece of evidence we have that these rod monochromats have cones as their high-level photopic receptors.

But what of the chemicals in the cones?

If we accept the evidence of Dowling on the rat and of Fuortes, Gunkel and me on man, which has been given earlier in this meeting, then the rod threshold change in dark adaptation is closely linked to the regeneration of rhodopsin. And we may readily account for the rapid cone branch of the dark adaptation curve and the slower rod branch, for the times of recovery in each case correspond closely to the times for regeneration of the corresponding visual pigments measured physically.

But this makes it rather surprising that in the "rod monochromats" of Alpern and Falls the cone and rod branches of the dark adaptation curve exhibit the normal time courses, if both receptors contain rhodopsin only, as they claim.

Perhaps before speculating upon the peculiar properties of "cone rhodopsin" which has the kinetics of a cone pigment and the spectrum of a rod pigment, we should like to be even more strongly assured that it exists. This paper of Alpern and Falls is the most thoroughgoing and diverse investigation of rod monochromats that has ever been published, but even here we are not quite sure that the receptors which give the Stiles-Crawford effect are the same as those which exhibit something close to rhodopsin sensitivity at moderate light levels. Nor is it easy to be sure that the rhodopsin-like sensitivity might not arise from a suitable fusion of blue and green cone sensitivities or pigments.

There is every reason to hope that the continuation of this fine work will bring a definite answer to these questions, as it already has to the "daylight rod" hypothesis.

Dr. Herbert Kaufman (Boston): Despite a natural reluctance to report someone else's data, there is valuable information that applies here. Dr. Raymond Harrison has been studying two brothers who appear to have typical rod monochromacy. One of the brothers met a violent end, and despite some autolysis, the eyes were obtained and were examined by Drs. Harrison and Cogan. Cones were present in the sections. The cones seemed to be abnormal and reduced in number; but, despite the fact that they did not appear normal, cones were certainly present. Dr. Harrison plans to publish the details of these cases.

Dr. Goodman, as I remember it, had data suggesting that there may be degrees of rod monochromacy. If this is true even the histology of this condition might be difficult to interpret.

Dr. Harold Falls (closing): I beg anyone who has in his practice individuals with monochromatism to plead with them to will their eyes to science. We need this badly.

Secondly, I want to emphasize one thing that I think was quite apparent in our presentation—that we have dealt with five individuals who to the best of our ability to ascertain do have the same amount of monochromatism but who do have some individual differences, showing, shall I say, a difference in response of that individual's makeup to a specific gene action.

Lastly, evidence is now forthcoming that we must very carefully investigate the family of the individuals we are studying, to ascertain whether or not (as in our particular case) these individuals show an auto-somal recessive form of the entity. There is pretty good evidence now, supported by Blackwell and Blackwell and collaborated by me in the investigation, that there is another form of monochromatism which we call blue monochromatism, which is sex-linked recessive inherited and therefore must have an entirely different mechanism or a different enzyme mechanism.

First, I plead for a careful investigation of families to be certain that the monochromatism one is dealing with is the specific entity we are talking about.

Second, I plead that you insist on the necessity of getting microscopic studies of these individuals.

Dr. Mathew Alpern (closing): First, let me say on behalf of Dr. Falls, Mr. Lee, and myself that it is an honor and a privilege to have such a distinguished discussant as has been our good fortune this morning. We would like to thank Dr. Rushton for his extensive analysis of this work.

A term like "rod monochromate" is not a very appropriate term. I would like to suggest that we no longer use this term. If this paper has any value at all, it shows that these people do have cones. These eyes are not cone blind. I think it confuses the issue if we continue to use these words, and I would suggest "typical total color-blindness" or "typical total monochromacy" or something of that sort as being much more suitable.

I think the remarks by Dr. Kaufman are extremely important. I am sure there are very large individual differences in these observers, that there are a great number of different kinds of monochromacy. I don't know how many different kinds there are, but I think this is one of the reasons for the confusion that exists in the literature.

THE FINE STRUCTURE OF BOWMAN'S LAYER AND THE BASEMENT MEMBRANE OF THE CORNEAL EPITHELIUM*

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INTRODUCTION

Bowman first described the membrane that bears his name in 1847. Later anatomists, including Fuchs and Salzman, essentially confirmed this work. Beginning with the work of Lowenstein in 1940, the presence of a basement membrane of the corneal epithelium was noted. Further differentiation on the histochemical level was carried out by Busacca and Redslob, Vidal, Wislocki, Teng and Katzin, La Tessa, and Calamettes. The electron microscopic appearance of the cornea has been studied by Sebruyns, Jakus, Sheldon, Garron and Feeny. In order to evaluate pathologic changes in the cornea, we felt it necessary to further define the fine structure of the anterior cornea.†

METHOD

The material used in this investigation consisted of five human eyes and two monkey eyes. Four of the human eyes were enucleated for malignant melanoma of the posterior pole and one for hemangioma. They were fixed within minutes of the time of enucleation with buffered osmic acid, dehydrated in alcohols and embedded in methacrylate. The monkey eyes were fixed in vivo and then enucleated. The tissue was cut on a Porter-Blum microtome and the sections were examined with RCA Model EMU 2b or 2e electron microscopes.

RESULTS

The basement membrane of the epithelium is found in man as a distinct layer lying between the basal cells of the corneal epithe-

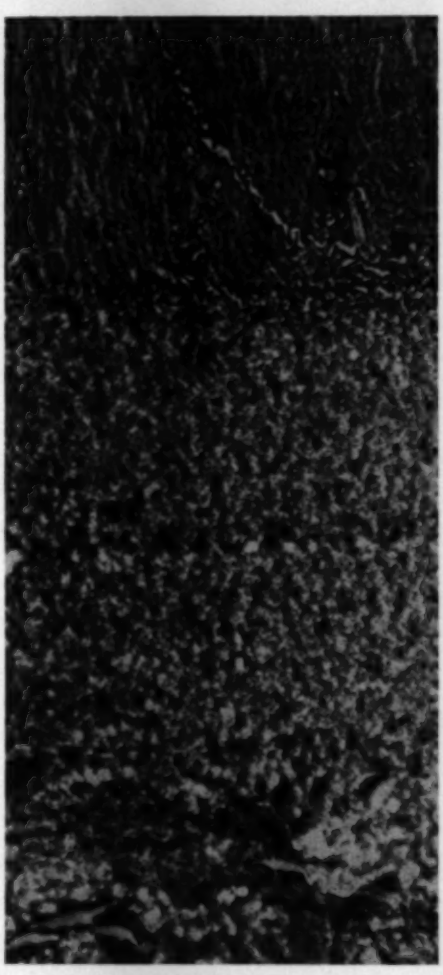

Fig. 1 (Kayes and Holmberg). Survey of human cornea, (BC) basal cells (BM) basement membrane (BL) Bowman's layer (S) stroma: ×3,300.

* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine. This investigation was supported in part by Research Grant B-1365, from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service. The research relating to this study was also financed in part by a grant from the Knights Templar Eye Foundation, Inc.

† Jakus has recently presented similar material at the VII International Anatomical Congress, April 1960.

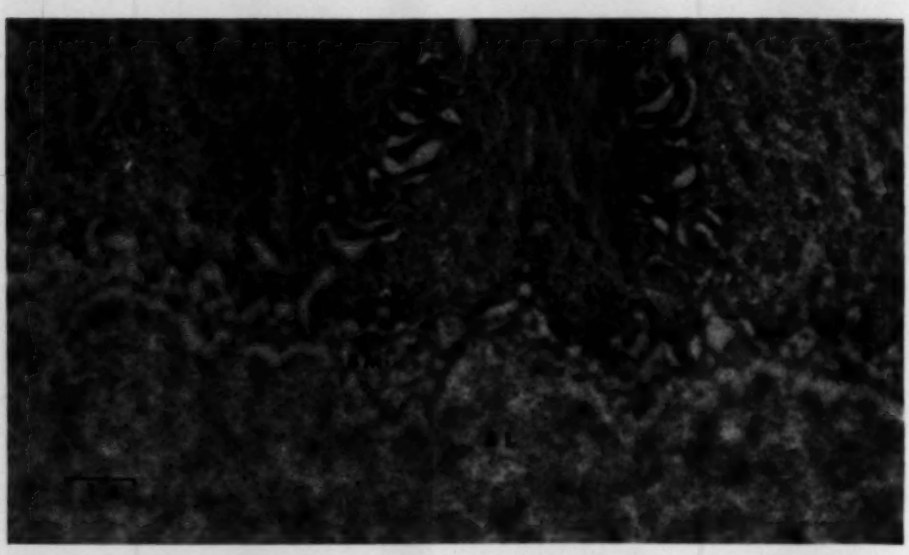

Fig. 2 (Kayes and Holmberg). Human cornea (BC) basal cells (BM) basement memberane (BL) Bowman's layer: ×14,000.

lium and Bowman's layer. The basal cells have an undulating surface for their posterior border. This appears more regular in the central parts of the cornea than it does toward the periphery. The peripheral basal layer resembles, somewhat, the interdigitations of the intercellular borders, but never becomes quite as involved (fig. 1, 2).

The basal cell membrane is seen as a distinct electron dense layer about 80 Å (ranging from 65 to 100 Å). At irregular intervals along the membrane (.15 u.-.6 u.) there appear to be thickenings (100 to 150 Å) of a material more electron dense than the rest of the membrane.

The basement membrane is about 0.15 to 1.5 u. thick. It appears as a meshwork of electron dense material surrounding apparently empty areas. There is no constancy in size of these components. No distinct fibers or organization could be seen within the basement membrane, nor did any fibers cross it.

For the most part, the anterior surface of the basement membrane is much less clearly defined than the basal cell membrane and is less electron dense. It is more irregular and is broken in many places. Like the basal cell membrane there are areas of greater electron density along its surface. These more dense areas, for the most part, lie directly opposite the more dense areas of the basal cell membrane (fig. 2, 3).

The posterior surface of the basement membrane is more irregular than the anterior. It is characterized by many small projections going into Bowman's layer. There appear to be differences in the density of this surface but for the most part not as marked as those of the anterior surface.

Bowman's layer is the area lying between the posterior border of the basement membrane and the well organized stroma fiber bundles. In man, this varies from eight to 14 u. in thickness (fig. 1, 4).

The anterior surface of Bowman's layer is quite irregular, closely paralleling that of the posterior surface of the basement membrane. There appears an electron empty area between these layers. This varies in thickness from 100 to 10,000 Å. Occasionally areas of electron density are seen along the anterior surface of Bowman's layer but no membrane appears present. There is much less difference in electron density between the basement membrane and Bowman's

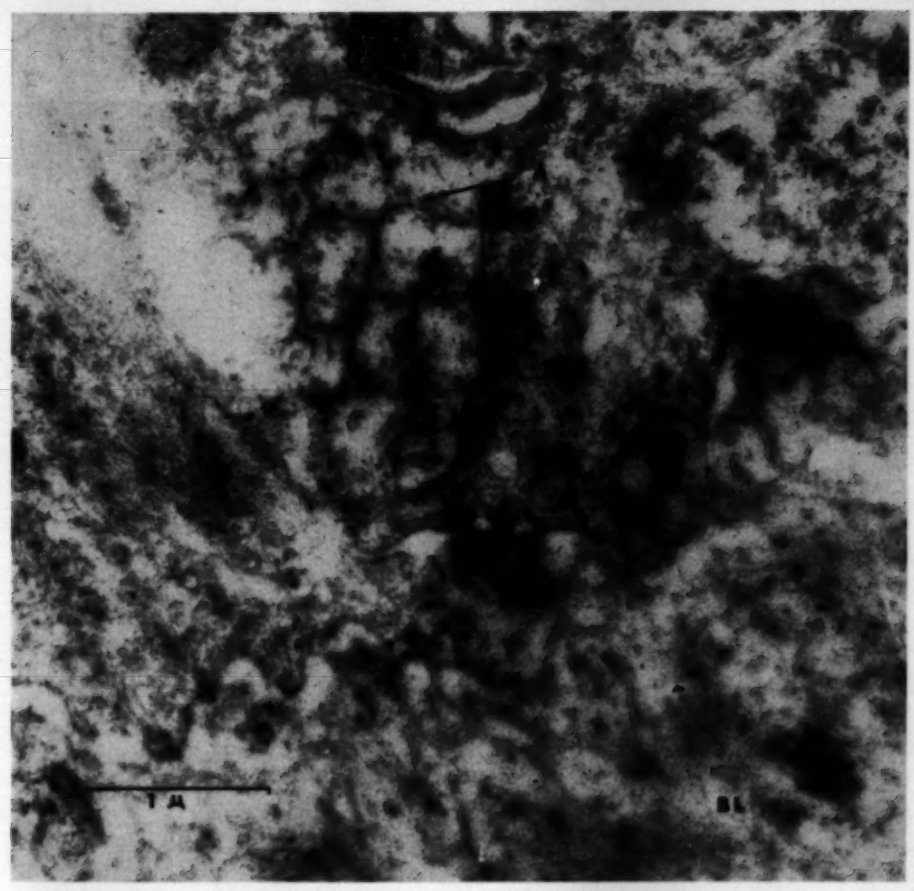

Fig. 3 (Kayes and Holmberg). Human cornea, (A) axons (BM) basement membrane (BL) Bowman's layer: ×36,000.

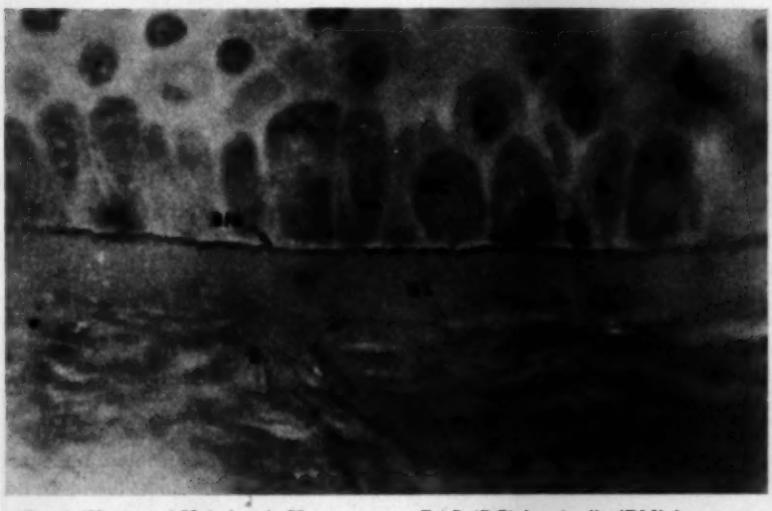

Fig. 4 (Kayes and Holmberg). Human cornea, PAS (BC) basal cells (BM) basement membrane (BL) Bowman's layer (S) stroma: ×630

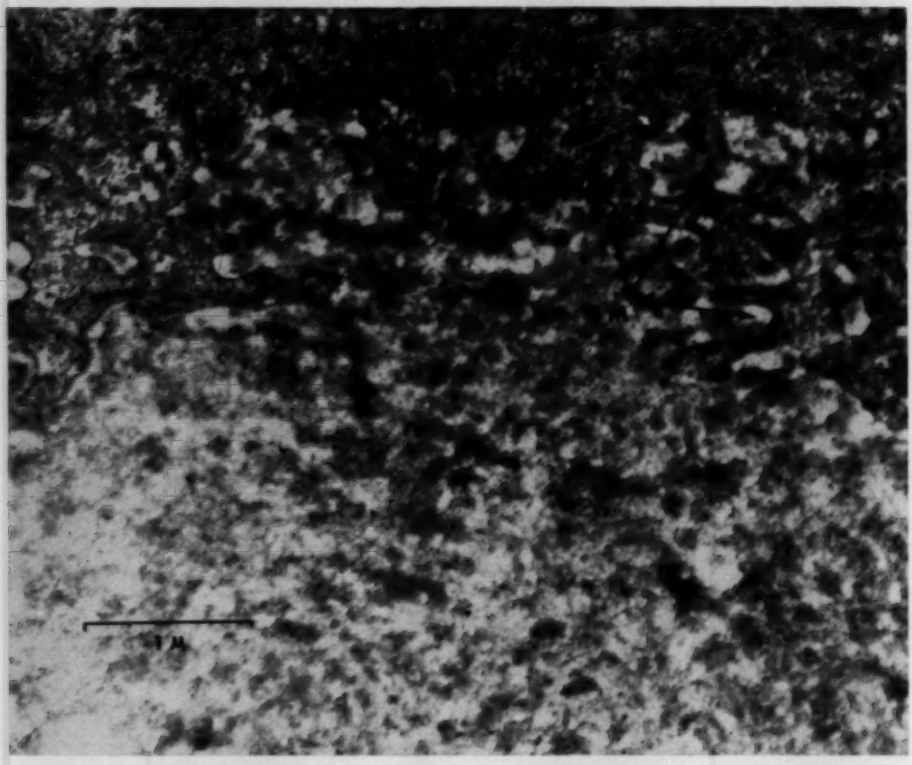

Fig. 5 (Kayes and Holmberg). Human cornea, tangential section (BM) basement membrane (BC) basal cell: ×31,800.

layer than there is between the basement membrane and the basal cell membrane.

In sections cut tangentially to the surface, the anterior basement membrane is easily delimited due to the clearly defined basal cell membrane. Many islands of basement membrane can be seen surrounded by the basal cells. These islands show the same electron-dense, structureless material that is seen in the sections cut perpendicular to the surface. Here again are the areas of greater electron density juxtaposed. No fibers appear to go across membrane surfaces (fig. 5, 6).

In the tangentially cut sections, the borders between the basement membrane and Bowman's layer are impossible to determine exactly.

Bowman's layer contains many nonoriented fibers. Some sections show a few more longitudinally cut fibers than others. The random arrangement was true both for perpendicular and tangential sections. In the eyes studied, the fiber size is shown in Figure 7.

A few fiber clumps of greater size (0.1 u.) were noted in the more peripheral areas. In addition to the fibers, there appears to be a structureless electron dense material filling in much of the space between the fibers. There also appears to be many very fine filaments present throughout this area.

The posterior border of Bowman's layer is much less distinct than the anterior border (fig. 1, 8). This region is marked by well defined bands of oriented fibers entering an area of unoriented fibers. These fiber bundles from the stroma contain fibers arranged parallel to each other. In the more posterior

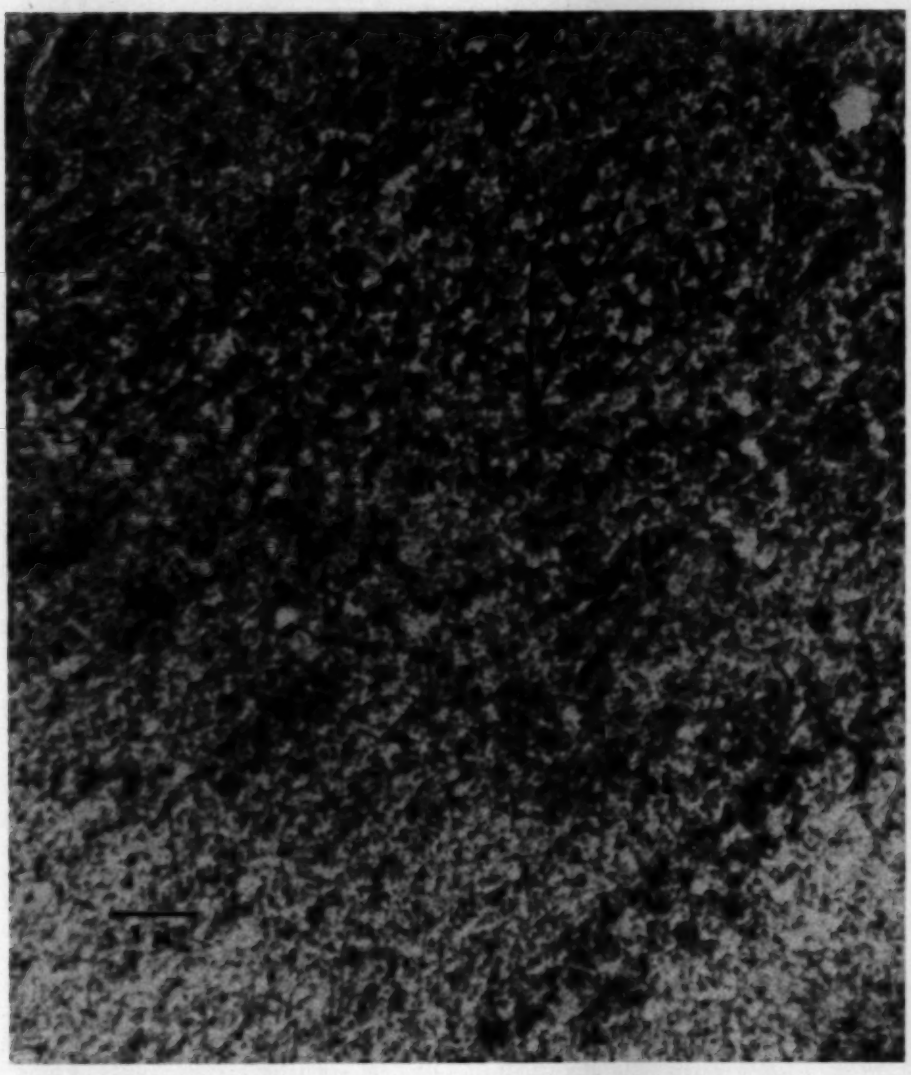

Fig. 6 (Kayes and Holmberg). Human cornea, tangential section (BM) basement membrane (BC) basal cell (BL) Bowman's layer: ×14,500.

layers the bundles are parallel to the surface and almost at right angles to each other. In the border zone some of the fiber bundles are seen to go almost at right angles to the surface. No cellular elements are noted in Bowman's layer. In two specimens, unmyelinated nerve fibers were noted in the posterior portion of the basal epithelial cells (fig. 3). By chance, they were not seen crossing either the basement membrane or Bowman's layer.

To date the findings for the rhesus monkey have closely paralleled those in man. A well defined basement membrane of the corneal epithelium and a Bowman's layer like that of man but somewhat thinner is present.

BOWMAN'S LAYER

Cross Section

Round	260-360 Å
Long	260-360 Å

Tangential Section

Round	250-360 Å
Long	300-360 Å

STROMA

Round	330-416 Å
Long	330-480 Å

Fig. 7 (Kayes and Holmberg). The noted fiber sizes represent 3000 measurements and no statistically significant differences were found.

Further work is in progress on the monkey cornea (fig. 9).

Comment

In 1847, Bowman delivered a series of lectures on the anatomy of the eye. The following passage is an excerpt: "The anterior elastic lamina has not hitherto, as far as I know, been distinguished by anatomists, and yet it seems a structure of a very interesting kind—it is a continuous sheet of homogeneous membrane, nearly similar in essential character to the posterior elastic lamina of the cornea and the capsule of the lens, being perfectly transparent and glassy, without appearance of internal structure." A similar description was made at the same time by Reichart.

In the ensuing years, little was added to our knowledge of this area. In 1940, Lowenstein, studying cases of Groenow's dystrophy, was surprised to find that the anterior part of Bowman's layer showed a slightly different staining reaction with Giemsa stain.

In 1949, Busacca and Redslob stained the basement membrane of the corneal epithelium with Mallory stain and clearly differentiated it from Bowman's layer.

Sebruyns, in 1951, examined the cornea with an electron microscope but using different techniques.

Vidal, in 1951, characterized the basement membrane, histochemically; and in 1952 Wislocki, working with the monkey eye noted a differential staining in the anterior part of Bowman's layer.

Teng and Katzin, in 1953, described the basement membrane in man and in several species of mammals which have no Bowman's layer. In 1954, with La Tessa, they characterized it further histochemically and noted, that besides lipids and polysaccharides, it also stained for reticulum with Wilders stain.

Jakus, in 1954, described the fine structure of the rat cornea as having a basement membrane of about 300Å thick; no Bowman's layer was seen in the rat. Sheldon reported similar findings in the mouse with the exception of a basement membrane of 600 Å.

In a paper on human cornea in 1954, Jakus described the fine structure of Bowman's layer in cross section as having unoriented fibers of about 160 Å and stromal fibers measuring 230 Å. No mensuration of the basement membrane was included.

In 1956, Calamettes et al. reported on the histochemistry of Bowman's membrane and the basement membrane, noting in Bowman's the similar staining reactions of the corneal stroma. In his paper, he further suggested the possible attachment of basement membrane to the basal cells by fiber bundles.

Graumann and Rohen did further work on the histochemistry of the cornea in 1958.

It is quite interesting that it took one hundred years for eye anatomists to recognize the layer that Bowman described in 1847, actually consisted of two separate entities. The basement membrane of the corneal epithelium appears to be a constant finding in all species of mammals studied to date. Its appearance in the electron microscope seems to differ in man and the primates from that found in the rat, mouse and rabbit (Holmberg 1959). The staining characteristics are very similar. It is interesting

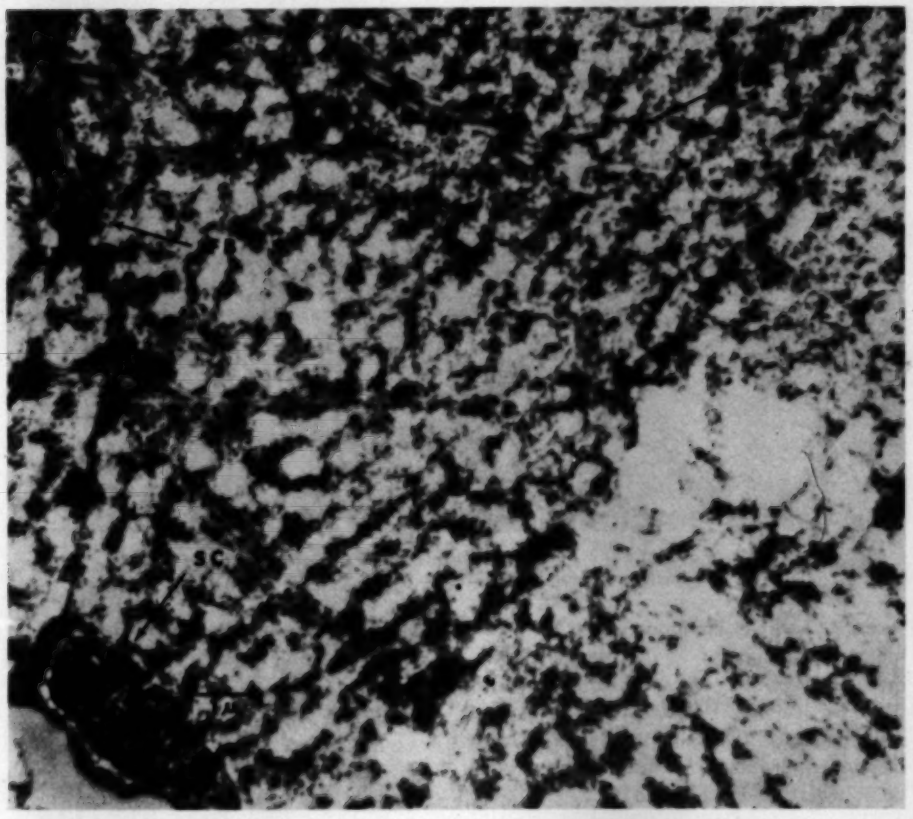

Fig. 8 (Kayes and Holmberg). Human cornea, (BL) Bowman's layer (FB) fiber bundles (SC) stroma cell: ×11,900.

to note that the basement membrane of the cornea and the internal limiting membrane of the ciliary epithelium resemble each other quite closely in their wide bands of homogeneous material and meshwork-like appearance. This differs quite markedly from the basement membranes found in the rat, mouse and rabbit where they appear much thinner and somewhat more dense.

Bowman's layer appears to have the same fiber arrangement in both cross and tangential sectioning. In situ measuring of fiber size in Bowman's layer presented a problem since the fibers are unoriented. The fibers seem to be round when examined in cross section; however, as one tries to measure more obliquely placed fibers, only the smaller of the two dimensions would appear to be valid. Similar consideration is given to longitudinally placed fibers. Since the thickness of the sections (500 Å) gives ample opportunity to measure only parts of the fibers rather than the whole, the thickest part seems to be the most reasonable one to measure. The stromal fibers appear to be slightly larger than those of Bowman's layer, but there appears to be considerable overlapping and the differences are not statistically significant.

Nowhere did we see fibers actually crossing from Bowman's layer into the basement membrane, or from basal cells into the basement membrane. However, the juxtaposed electron dense areas involving the above sur-

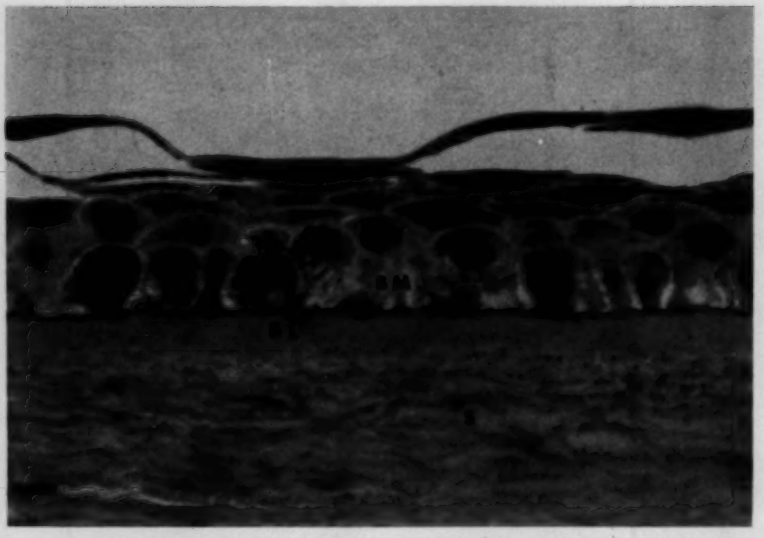

Fig. 9 (Kayes and Holmberg). Monkey cornea, PAS (BC) basal cells (BM) basement membrane (BL) Bowman's layer (S) stroma: ×630.

faces, as well as the intercellular surfaces, might be thought of as desmosomes. Since most of the studies on corneal cohesion antidated the use of the electron microscope and histochemical differentiation of the basement membrane, it would be most interesting to know where breaks occurred.

The histochemical similarities of the substantia propria and Bowman's layer, as well as the electron microscopic similarities, would make it seem that Salzman's concept of Bowman's layer as a specially modified layer of the anterior stroma is a correct one. Bowman's layer is most fully developed in man and the higher apes, but it does appear in a less well developed fashion in the lower monkeys and in other mammalian species. It must, in any case, not be considered a membrane.

It is hoped in the near future to apply the knowledge gained on the normal corneal structure to pathologic states, especially keratoconus and the corneal dystrophies.

Summary

The ultrastructure of the basement membrane of the corneal epithelium and Bowman's layer as seen with the electron microscope, have been described. Their relationship and connections to neighboring structures in cross section and in tangential section has been described and discussed.

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Discussion

Dr. George D. Pappas (New York): In the manuscript submitted to me, Drs. Kayes and Holmberg substantiate the distinct anatomic and histochemical differentiation between Bowman's layer and the basement membrane of the corneal epithelium. It is difficult to get good preservation for electron microscopy of human eye tissue, since the eye can only be fixed after enucleation. For that reason, much of the detail one can find in well-preserved experimental animal material is missing in the electron micrographs shown.

However, even though present methods of fixation of human eye tissues are unsatisfactory, it is still essential to do comparative studies such as Jakus and others have done. Bowman's layer, for example, is most fully developed in man. Also, the trabecular meshwork of the drainage angle in the human eye appears to differ from that of laboratory animals in which the eye can be fixed before enucleation.

The authors measured fibers both in Bowman's layer and in the stroma of the cornea. They concluded that there were no statistically significant differences between fiber thickness in these two areas. The question may be raised as to what the apparent fiber diameter similarities in these two areas signify?

In addition, were the authors able to compare the density of the collagen fibers per unit area in the stroma with that of the fibers in Bowman's layer?

Dr. J. Reimer Wolter (Ann Arbor, Michigan): I saw in the pictures the axon bundles that Dr. Kayes pointed out underneath Bowman's membrane and the basement membrane. It is very well known that these nerve fibers penetrate these layers and enter the epithelium. I wonder whether the authors have seen these nerve fibers penetrate these layers, and I wonder why Dr. Kayes could observe them enter into or between the epithelium.

A question that is still being discussed in whether these nerve fibers in the epithelium do enter the epithelial protoplasm or whether they remain between the epithelial cells.

Dr. Jack Kayes (closing): I would like to thank the discussers for their comments. We have not measured the per unit density of the fibers in Bowman's layer and in the stroma.

To answer Dr. Wolter's question, it was our feeling that the axons appear to be surrounded by their own mesaxons rather than by the cells, but we cannot be sure from the micrographs. We are doing further work in this area.

We have not actually seen one of the axon bundles crossing Bowman's layer, but I think this is merely a chance finding and that sooner or later we will see it.

We are carrying on further work now in eyes with keratoconus and with corneal dystrophy in an attempt to use the information we have gathered here to some good end.

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